The Role of Multiple Sclerosis-Associated Genetic Risk Factors in Shaping Epstein-Barr Virus Specific Immune Control

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Summary

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease believed to be mediated by autoreactive $CD4^+$ T cells, however the exact disease mechanisms are not known. More than 200 genetic risk factors and several environmental risk factors have been associated with the disease. Amongst genetic risk factors, the MHC class II molecule HLA-DRB1*15:01 (HLA-DR15) confers the strongest risk. In addition, >200 single nucleotide polymorphisms (SNPs) have been associated with MS risk, mostly mapping to genes involved in adaptive immunity and T helper cell pathways. Epstein-Barr virus (EBV) is a ubiquitous virus infecting the majority of the human adult population. Even though in the majority of cases the infection goes unnoticed, in a subset of young adults the virus can cause an acute infection known as infectious mononucleosis (IM). Interestingly, strong evidence now supports EBV infection to be an environmental risk factor for MS and to synergize with the HLA locus to further increase this risk. The interactions between MS genetics and the environment remain to be understood.

In this PhD thesis, we aimed to investigate the interaction between MS-associated genetic risk factors and EBV using our humanized mouse model of EBV infection. We have demonstrated that animals reconstituted with donors positive for HLA-DR15 show higher basal activation in the T cell compartment. Upon EBV infection, we saw higher frequencies of $CD8^+$ T cells and higher numbers of activated T cells. Despite this, the animals displayed higher EBV viral loads, suggesting defects in EBV immune control. Upon performing T cell cloning from these EBV-infected mice, we observed that HLA-DR15-restricted $CD4^+$ T cell clones demonstrated cross-reactive responses towards allogeneic lymphoblastoid cell lines when compared to HLA-DR4-restricted clones. To develop on these results, we next aimed to create an HLA-DR15 transgenic humanized mouse model, which we could use to study these responses further. While we saw HLA-DR15 expression in mouse tissues, these animals did not reconstitute human immune cells, possibly due to incorrect insertion of transgene and activation of the murine myeloid cells. We will next breed them to the murine MHC class II-deficient NSG mouse strain and examine effects on reconstitution in this setting.

Finally, we aimed to investigate MS-associated risk variants, their causality and effect on T cell responses upon EBV infection. Even though individually these variants only slightly increase the risk of MS, they likely act together in linked pathways interacting with the HLA locus and environmental factors. Upon short-listing several candidates, we developed a SNP-typing method of our donors. We reconstituted animals with donors homozygous positive, negative or heterozygous for the SNP downstream of the EOMES gene, the transcription factor

involved in T and NK cell cytotoxicity and effector functions. In these animals, we did not observe differences in bulk T cell expression of EOMES at steady state or during EBV infection. We further want to investigate this in T cell clones and include additional risk variants.

Together, we have provided some evidence into the interaction between MS-associated genetic and environmental risk factors. HLA-DR15 might prime for more cross-reactive specificities, while single risk variants could act to decrease the threshold of T cell activation and immune dysregulation. As a result, HLA-DR15 restricted T cells might less efficiently control EBV infection, lead to higher immune activation and expansion of autoreactive lymphocytes, which could lead to the development of MS.

1 Introduction

1.1 Epstein-Barr virus

1.1.1 Discovery

After successfully culturing tumor cells from Burkitt's lymphoma (BL) *in vitro* (Epstein and Barr, 1964), Epstein discovered the first human tumor virus, Epstein-Barr virus (EBV), when observing virus particles in the cytoplasm of these lymphoblast cell lines (LCLs) (Epstein *et al.*, 1964). In a study a few years later, Henle and colleagues demonstrated that cultured cells from BL harboring EBV managed to induce the growth of peripheral leukocytes of a healthy donor (Henle *et al.*, 1967).

Jondal and Klein discovered EBV receptors on B cells, and Pattengale and colleagues additionally found that EBV infects B cells and transforms them into proliferating B lymphoblasts (Jondal and Klein, 1973; Pattengale *et al.*, 1974). Twenty years later, it was shown that the B cell is the site of the full EBV life cycle (Thorley-Lawson *et al.*, 1996). In addition, >90% of persistently EBV-infected cells in the peripheral blood of the asymptomatic host are in fact resting, non-activated B cells, proposing this to be the cell of long-term persistence (Miyashita *et al.*, 1997). However, even though in a latent state, the virus actively replicates and sheds into the saliva. Immunohistology suggested that, in acutely-infected patients, the replication of the virus occurs in B cells found in the epithelium of mucosal lymphoid tissues (Anagnostopoulos *et al.*, 1995). In 1998, it was then demonstrated that EBV infection *in vivo* likely follows antigen-driven activation. Whilst in lymphoid tissue EBV infects naïve and memory B cells, EBV-infected cells in the periphery are in fact memory B cells (Babcock *et al.*, 1998).

1.1.2 Mode of infection

EBV is one of eight viruses in the family of known human herpesviruses and together with Kaposi's sarcoma-associated herpesvirus (KSHV), it belongs to the gamma herpesvirus subfamily (Hislop *et al.*, 2007). It carries a large, double-stranded DNA genome which is 172 kilobase pairs long (Yates *et al.*, 1985).

To infect B cells, EBV uses its glycoprotein (gp) gp350 (BLLF1) to bind to the complement receptors CD21 or CD35 and also gp42 (BZLF2) binding to MHC class II

molecules. Targeting integrins by gH (gp85) and gL (gp25) then promote viral fusion with the cell membrane that is executed by the gB (gp110) protein (Shannon-Lowe and Rowe, 2014).

EBV and the human host have developed a fine balance which allows for their mutual coexistence. The virus has two stages of life cycle - lytic and latent infection. Following oral transmission to a naïve host, EBV is probably delivered across the polarized mucosal epithelium by transcytosis (Tugizov *et al.*, 2003; Tugizov *et al.*, 2013). Interestingly, Dunmire and colleagues found EBV viral loads in the oral cavity only five to six weeks post primary EBV infection and before the onset of symptoms, suggesting high levels of lytic replication not taking place within epithelial cells. Instead, their data suggest that B cells could be the major cell type infected in the nasopharyngeal sites and disseminating into peripheral blood after oral transmission. This is reflected by low levels of viral genomes measured in the peripheral blood prior to the oral cavity. A few days before acute disease onset, high viral loads were detected in the oral wash, possibly resulting from viral reactivation in latently infected B cells and subsequent infection of epithelial cells (Dunmire *et al.*, 2015).

Upon B cell infection, EBV switches into the latent growth-transforming program. The infected B cell pool is expanded and in acute disease represents up to 10% of all B cells in the peripheral blood (Hislop *et al.*, 2007). Even though most of the infected B cells are eventually removed by the immune system, some cells downregulate antigen expression and enter the memory B cell pool. In this way, the virus persists for the life of the individual. The recirculating B cell population may receive stimuli such as antigenic stimulation or signals for plasma cell differentiation and periodically switch back to the lytic cycle to produce and spread infectious virions to a new host (Hislop *et al.*, 2007) (Fig. 1). Transmission is further improved by lytic replication in a permissive cell type such as specialized mucosal epithelial cells (Shannon-Lowe *et al.*, 2006) and the derived virus is additionally more efficient in infecting B cells in the new host (Borza and Hutt-Fletcher, 2002; Hutt-Fletcher, 2007). There are high amounts of viral shedding into the throat after symptomatic primary infection and this can persist in saliva for even longer than 6 months (Balfour *et al.*, 2005; Fafi-Kremer *et al.*, 2005). In addition, low levels of virus shedding into the saliva has been observed in the throats of long-term virus carriers (Balfour *et al.*, 2005).

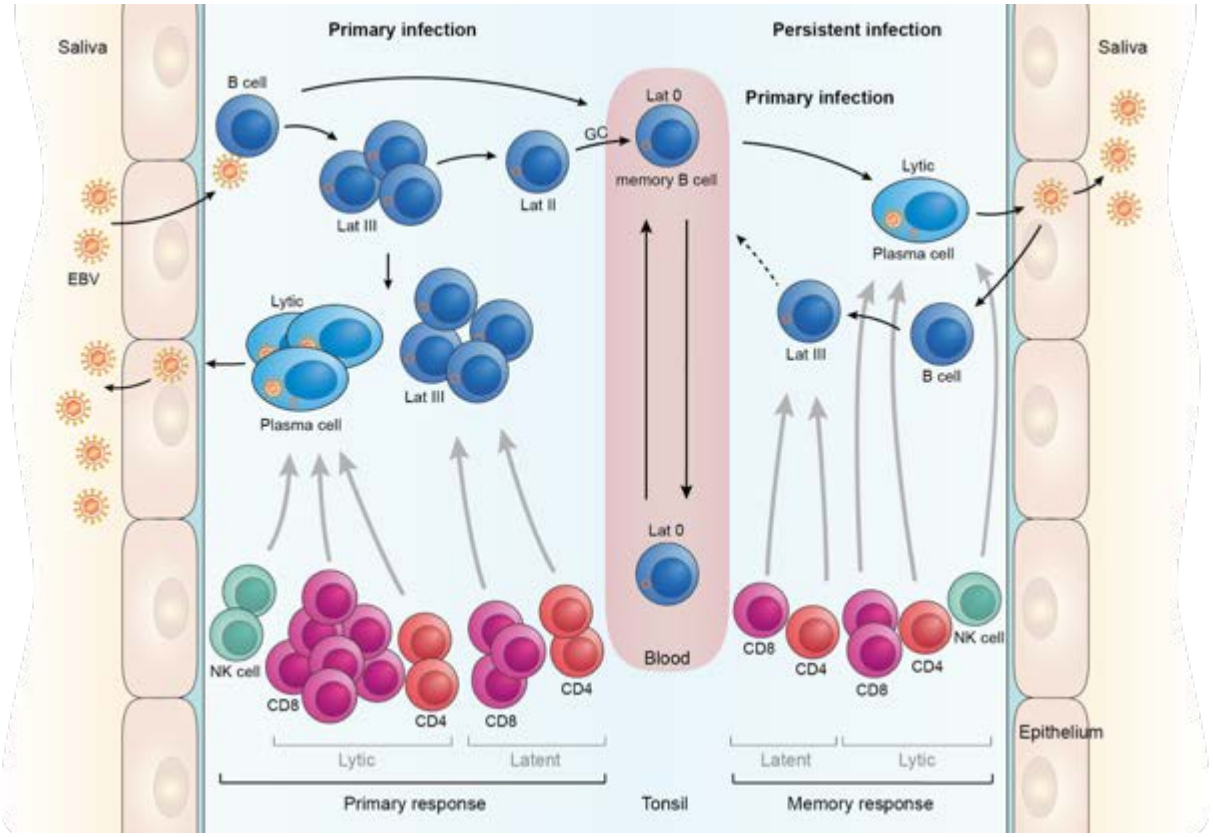

Figure 1: EBV primary infection and persistence. After oral transmission, EBV passes through the epithelial cell layer to infect local B cells in secondary lymphoid tissues and the tonsils and drives them into latency (Lat) III. If infected B cells are memory cells, they can directly enter latency 0. Latency III cells further differentiate via latency II through the germinal center to latency 0 memory B cells, which recirculate in peripheral blood. B cells can enter the lytic cycle early after infection, start virus reactivation and infect oral epithelial cells, which again release virions into the saliva. This primary response is largely controlled by $CD8^+$ and $CD4^+$ T cells to both latent and lytic antigens, as well as NK cells during lytic infection. From memory B cells in the circulation, differentiation into plasma cells reactivates the lytic cycle, infection of epithelial cells and subsequent release of virions into saliva, infecting a new host or adjacent B cells. During persistent infection, memory $CD8^+$ and $CD4^+$ T cells control both latent and lytic and NK cells lytic programs, however the response is much smaller than during primary infection.

(Adapted from (Taylor *et al.*, 2015; Munz, 2019).

1.1.3 Latent and lytic cycles

During the lytic cycle, the virus sequentially expresses three sets of proteins. First, the immediate early (IE) proteins serve as the transactivators of early gene expression. Next, early (E) proteins are expressed and consist of essential components of the viral DNA replication complex and finally, late (L) proteins form the structure of the virion (Hislop *et al.*, 2007).

The virus also carries genes which are crucial for it to successfully establish the latent state. The latently infected cells first undergo a phase of cell proliferation, which is associated with the transient expression of the viral latent genes. This allows for the expansion of the latently infected cell pool before viral genes are suppressed, holding a stable reservoir of viral-infected but antigen-negative cells (Hislop *et al.*, 2007). EBV's B cell growth-transforming ability depends on latent genes encoding six EBV nuclear antigens (EBNA) -1, 2, 3A, 3B, 3C and LP, as well as two latent membrane proteins (LMP) 1 and 2. All eight of these proteins are expressed in EBV-transformed LCLs *in vitro* (Hislop *et al.*, 2007).

EBV infection of epithelial cells is not very well characterized, however it is thought that epithelial cells can mainly support lytic replication, while both latent and lytic infection programs take place in B cells (Thorley-Lawson and Gross, 2004). In B cells, different latency programs depend on the differentiation stage of the infected cell. Infection of naïve B cells shows the expression of all eight EBV latent proteins in latency III. After entering the germinal center, B cells only express three latent proteins; EBNA1, LMP1 and LMP2, in latency II. In the latency I program, only EBNA1 is expressed in homeostatically proliferating infected memory B cells. Finally, non-proliferating infected memory B cells in the periphery are in the latency 0 state where no EBV proteins are expressed (Babcock *et al.*, 1998; Babcock *et al.*, 2000; Hochberg *et al.*, 2004). Lytic replication is induced during differentiation of infected memory B cells into plasma cells and results in the activation of the promoter for the gene triggering viral replication, BZLF1 (Laichalk and Thorley-Lawson, 2005).

The EBV antigen LMP-1 is a member of the TNFR superfamily and acts to mimic the B cell CD40 receptor. It assists antigen-stimulated B cells to enter the germinal center reaction after interaction with $CD4^+$ T cells to differentiate into memory B cells or antibody-secreting plasma cells (Farrell, 1998). LMP-1 plays a crucial role in human B cell transformation (Farrell, 1998) and is required for LCL growth *in vitro* (Hayward, 2004), however, EBNA protein expression is also needed for sufficient immortalization. EBNA2 is one of the first viral genes expressed after B cell infection and important for B cell transformation. It mimics function of intracellular Notch by binding to a transcriptional repressor downstream of Notch, converting it into a transcriptional activator and activating gene transcription of bound promoters. EBNA-LP acts as a positive regulator of EBNA2. On the other hand, the three EBV proteins EBNA3A,

3B and 3C compete with EBNA2 for binding the transcriptional repressor and thus blocking the transcriptional activation and dampening gene transcription. Like LMP-1, EBNA2 and EBNA3C are also essential for EBV immortalization of infected B cells into LCLs (Hayward, 2004).

EBNA1 is the only nuclear EBV protein expressed in both the latent and lytic programs (Sivachandran *et al.*, 2012). It is critical in maintaining the viral episome in infected, proliferating B cells by binding to the latent origin of replication, oriP (Yates *et al.*, 1985). Unlike lytic replication, which uses a viral DNA polymerase, the viral genome in latently infected cells uses the host cell replication machinery to replicate once per cell cycle and gets passed to daughter cells (Adams, 1987; Yates and Guan, 1991). EBNA1 binding of the oriP additionally effects the expression of other latency genes (Gahn and Sugden, 1995) and cell proliferation and survival (Kennedy *et al.*, 2003; Sivachandran *et al.*, 2008).

The differentiation of the infected memory B cell into a plasma cell can trigger the switch to the lytic cycle. BZLF1 is the first EBV lytic gene to be expressed (Laichalk and Thorley-Lawson, 2005). It transactivates its own promoter, Zp, and the other immediate-early promoter, Rp, inducing the expression of BRLF1 transcription factor (Miller *et al.*, 2007). Together, these two proteins then induce the expression of other EBV lytic genes (Feederle *et al.*, 2000). BZLF1 also binds to the origin of lytic DNA replication, oriLyt, and recruits viral DNA polymerase and factors that are needed for lytic replication (Lieberman *et al.*, 1990; Gao *et al.*, 1998).

1.1.4 Acute primary EBV infection – infectious mononucleosis

1.1.4.1 Discovery

In the 1880s, Nil Filatov, a Russian pediatrician, first recognized a unique illness (Filatov and Earle, 1904). A few decades later, Sprunt and Evans gave the disease its current name, infectious mononucleosis (IM), to the disease which presented itself as an acute infection causing fever, cervical lymphadenopathy and pharyngitis, as well as displaying atypical large lymphocytes in the peripheral blood (Sprunt and Evans, 1920). Even though the etiology of the illness was unknown, it later turned out to provide great advances in EBV research.

Similar to the experiments done with BL cell lines, peripheral blood lymphocytes of IM patients had the capacity to establish growing cultures (Pope, 1967) and additionally, their infiltrates induced the growth of fetal human lymphocytes *in vitro* (Pope *et al.*, 1968). This work suggested the presence of a viral agent involved in the proliferation, perhaps the same as

found in BL cells. This was further supported by work that detected EBV in all LCLs grown out from individuals in the acute phase or with a past history of IM (Diehl *et al.*, 1968).

The first serological evidence of a link between EBV and IM came from a study by Henle and colleagues in 1967, when to their luck a lab technician became EBV seropositive after contracting IM. As a result of this, sera from IM, as well as BL patients were examined and strikingly, 100% had, and mostly high, anti-EBV antibody titers and negative pre-infection sera. Controls were 20-50% positive with mostly low titers (Henle *et al.*, 1968).

These anti-EBV antibodies were additionally observed by indirect immunofluorescence (Niederman *et al.*, 1968), found to be complement-fixing (Gerber *et al.*, 1968), virus-neutralizing (Miller *et al.*, 1972), and they reached peak levels a few weeks after disease onset and persisted for years later (Niederman *et al.*, 1970).

Several efforts to isolate the virus from IM patients followed. EBV was found in the throat washings of IM patients, as well as EBV-seropositive, but not EBV-seronegative healthy donors and was able to transform cord-blood leukocytes (Gerber *et al.*, 1972; Miller *et al.*, 1973). More work has shown that the virus is found in the throat of healthy individuals without a past history of IM, as well as individuals receiving immunosuppressive therapy. Whilst in IM patients, EBV is present in the throat and oropharynx several weeks or months after clinical illness (Miller *et al.*, 1973; Niederman *et al.*, 1976), EBV presence in the throat of healthy individuals without a past history of IM and/or individuals receiving immunosuppressive therapy (Strauch *et al.*, 1974) hinted towards a reactivation of the latent virus from oropharyngeal sites. Work by Niederman and colleagues was able to demonstrate that oral fluids contain extracellular EBV, suggesting a cell type in which the virus undergoes its full life cycle. They demonstrated with an early IM patient that saliva was invariably positive with the virus, whilst other oral sites were intermittently positive. Infectious virus was present in low titers and excreted intermittently both in patients with IM and healthy individuals, which together offered an explanation for the moderate contagiousness of this infection and widespread and continuous transmission by intimate contact (Niederman *et al.*, 1976).

1.1.4.2 Epidemiology and clinical manifestations of the acute illness

EBV is an extremely common agent and has a worldwide distribution (Niederman, 1982). When EBV is acquired early in childhood, it usually remains as an asymptomatic primary infection (Biggar *et al.*, 1978a). How EBV is transmitted at this early age is not known, however a likely explanation is infection by parents or siblings, who have periodic EBV shedding into their oral secretions (Sumaya and Ench, 1986). A study of Melanesian children found EBV acquisition very early in infancy, suggesting its spread by interpersonal contact with

several carriers, such as pre-chewing food for babies and infants (Lang *et al.*, 1977). Extremely early acquisition of primary EBV infection has also been shown for example in newborns in Accra, Ghana, where 81% of infants were infected by the age of 21 months (Biggar *et al.*, 1978b; Piriou *et al.*, 2012).

An appropriate immune response to EBV is behind the fine balance that exists with its human host. EBV-associated diseases or pathologies mostly arrive either when an individual is severely immunocompromised or if an exaggerated, hyperactive immune response is mounted to the virus (Hislop *et al.*, 2007).

IM is a well-recognized disease in the developed world with advanced sociohygienic standards, with peak incidence occurring in adolescents and young adults. In up to 25% of cases, mostly in the West, primary EBV infection can be delayed until early adulthood (Crawford *et al.*, 2006). When EBV transmission happens later in life, primarily following deep kissing, it is then able to cause this acute illness (Hoagland, 1955; Niederman, 1982; Balfour *et al.*, 2013).

The incubation period of EBV before the onset of IM symptoms is 33 to 49 days (Hoagland, 1955; Svedmyr *et al.*, 1984), with most frequently-experienced symptoms of sore throat, cervical lymphadenopathy, fatigue, upper respiratory track symptoms, head ache, decreased appetite, fever, body aches and abdominal pain. The illness lasts between 3 and 66 days, however viral shedding has been observed prior to symptoms in the majority of patients. Oral cell pellets and whole blood lasted EBV DNA-positive for a mean of 272 and 95 days, respectively (Balfour *et al.*, 2013; Dunmire *et al.*, 2015).

It is still not completely understood why IM is largely a disease of adolescents and young adults and why primary infection sometimes presents as asymptomatic. First, it is possible that in preadolescents, the disease is simply not recognized. The heterophile antibody test, a routine IM diagnostic test, has shown to be unreliable in children under the age of four years. Evaluating a peripheral blood smear for atypical lymphocytes characteristic of IM, followed by EBV-specific assays, has proven more successful in identifying IM in young children (Horwitz *et al.*, 1981).

Secondly, during the acute and convalescent stages of IM, patients can have between 8 000 and >1 000 000 EBV copies / ml of saliva up to 6 months post diagnosis, versus 0 – 10 000 EBV copies / ml of saliva in healthy EBV carriers (Fafi-Kremer *et al.*, 2005; Hadinoto *et al.*, 2008; Balfour *et al.*, 2013). Another possibility could therefore be that large amounts of infectious virus are transmitted by deep kissing, where on the other hand, only small infectious inoculums are transmitted from asymptomatic parents or siblings. A third and fourth possible

reason is an altered $CD8^+$ T cell and natural killer (NK) cell-mediated immune control of primary infection.

1.1.4.3 $CD8^+$ and NK cells in IM

There is evidence from IM patients (Williams *et al.*, 2005; Balfour *et al.*, 2013), primary human immune deficiencies (Benoit *et al.*, 2000; Parolini *et al.*, 2000) and humanized mouse models (Strowig *et al.*, 2009; Yajima *et al.*, 2009; Chijioke *et al.*, 2013) to suggest that both NK cells, and T cells, play a role in EBV control.

One possible reason for IM in adolescence is the difference in the $CD8^+$ T cell response mounted against the transmitted virus in adolescence and early adulthood. A study by Clute and colleagues found influenza-specific memory $CD8^+$ T cells cross-react with EBV (Clute *et al.*, 2005). However, in a study published a few years later, even though the authors saw bystander activation of influenza-specific memory $CD8^+$ T cells during IM, these cells did not expand. Hence, the $CD8^+$ T cell expansion was composed mainly of latent and lytic EBV-specific T cells (Steven *et al.*, 1996; Steven *et al.*, 1997; Hoshino *et al.*, 1999; Odumade *et al.*, 2012). Whether these $CD8^+$ T cells play a pathologic or protective role in IM is unclear. A reduction in activated, EBV-specific $CD8^+$ T cells was parallel to a drop in EBV genome load in PBMCs, suggesting an effective clearing of virus-infected cells (Hoshino *et al.*, 1999). However, in a special cohort of donors undergoing primary asymptomatic EBV infection, the seroconversion occurred in the presence of normal levels of $CD8^+$ T cells, as opposed to the expected significant lymphocytosis seen in IM patients. Additionally, the large expansion of $CD8^+$ T cells observed in IM patients was largely clonal in composition, indicating antigen-driven amplification (in one IM patient, 44% of circulating $CD8^+$ T cells recognized a single epitope (Callan *et al.*, 1998)) (Annels *et al.*, 2000). In contrast, asymptomatic seroconverters showed regular, Gaussian-like profiles in the TCR repertoire throughout the course of infection until persistence. Surprisingly, both IM patients and asymptomatic seroconverters had high cell-associated EBV viral loads (Silins *et al.*, 2001).

Similar data was seen obtained in another cohort of asymptomatic primary infections. Together with the observations that while $CD8^+$ T cells did not rise to the levels observed in IM patients in asymptomatic infections, 40% of the T cells were activated and at least 15% of these cells were specific to one EBV lytic cycle epitope. Notably, one asymptomatic donor showed no $CD8^+$ T cell expansion or activation, nor detectable EBV-specific $CD8^+$ T cell responses during primary infection and three months later, despite high EBV loads in the blood. Only 16 months later was an EBV-specific $CD8^+$ T cell response detected (Abbott *et al.*, 2017).

This suggests that the T cells, when expanded beyond homeostasis in IM, may become counterproductive and immunopathogenic (Silins *et al.*, 2001) and that viral loads alone cannot drive symptoms, as asymptomatic infections tend to have similarly high viral loads as IM patients (Abbott *et al.*, 2017).

While the activation of NK cells, as determined by levels of granzyme B, was not significant, an expansion of NK cells in the blood of IM patients was observed. This coincided with $CD8^+$ T cell lymphocytosis, positively correlating with $CD8^+$ T cell numbers and disease severity (Balfour *et al.*, 2013). In an earlier study however, even though there was an elevation in NK cell numbers, which comprised an increased proportion of $CD56^{bright}$ NK cells (the dominant NK cell subset in lymphoid tissues) with an enhanced ability to lyse EBV-infected B cells, the NK cell numbers correlated inversely with viral loads (Williams *et al.*, 2005).

In vitro, this $CD56^{bright}$ NK cell subset proliferated and produced large amounts of IFNγ, which restricted EBV B cell transformation. Tonsillar NK cells were additionally more efficient than peripheral blood NK cells (Strowig *et al.*, 2008). Interestingly, using a NOD-scid $\gamma_c^{-/-}$ humanized mouse model of EBV infection, NK cells, mostly the early-differentiation phenotype subset $CD56^{dim}$ $NKG2A^+$ killer-cell immunoglobulin-like receptor $(KIR)^-$, were also increased during an IM-like disease and preceded the peak of the T cell response. They preferentially targeted lytically infected cells and upon their depletion, log higher viral loads were observed. Additionally, when NK cells were expanded and further differentiated, they controlled EBV infection less well, suggesting that NK cells with an early-differentiation phenotype are better at viral control (Chijioke *et al.*, 2013).

In line with this, a study by Azzi and colleagues has demonstrated a preferential proliferation of this early differentiated, $CD56^{dim}$ $NKG2A^+$ KIR^- NK cell subset in pediatric IM patients, versus in either adolescents or adults. This NK cell subset degranulates and proliferates on exposure to EBV lytic antigens and is seen to persist for several months after acute IM (Azzi *et al.*, 2014). This suggests that this early-differentiated NK cell subset could serve an important role early in infection by controlling lytic replication and limiting the number of infectious virus particles entering the B cell pool and also by reducing the $CD8^+$ T cell response by reducing available lytic antigens, its main drivers. Also, there could be an impaired NK cell control, as IM is a disease which shows high viral loads and an exaggerated T cell response. Individuals with asymptomatic infection did not seem to show the expansion of either total NK cells or of the $CD56^{dim}$ $NKG2A^+$ KIR^- NK cell subset. The authors however suggest that NK cell populations in the blood do not neccesarily reflect NK cell subsets and action in oropharyngeal sites (Abbott *et al.*, 2017). Nevertheless, as IM mainly manifests in adolescents and young adults, who have decreased frequencies of this early-differentiated NK cell subset, an age-dependent impaired NK cell-mediated immune control could be a factor contributing to

IM (Azzi *et al.*, 2014). However, the role of NK cells in the control of EBV transformation is debatable, as clinical evidence shows that patients receiving T cell-depleted stem cell transplants develop EBV-driven lymphoproliferative disease most commonly in the first 3 to 6 months after transplantation. In this time, NK cells are present while T cells are still absent (O'Reilly *et al.*, 1997).

1.1.5 T cell responses to EBV

As mentioned before, in acute disease, there is a vast expansion of EBV-specific $CD8^+$ T cells. Much progress has been made in deciphering EBV specificities in the blood of IM patients (Steven *et al.*, 1996; Steven *et al.*, 1997; Callan *et al.*, 1998; Catalina *et al.*, 2001; Hislop *et al.*, 2002; Pudney *et al.*, 2005). The most dominant responses in acute infection are specific for IE and E lytic cycle antigens, representing anywhere between 1% and 40% of total $CD8^+$ T cells. In addition, IE epitopes are most efficiently presented and recognized by effector $CD8^+$ T cells. As the lytic cycle progresses (IE > E > L), the efficiency in epitope presentation and immunodominance decreases (Pudney *et al.*, 2005). On the other hand, responses to latent antigens during acute infection are delayed and in lower frequencies, representing 0.1% to 5% of total $CD8^+$ T cells. The most dominant latent epitopes are from the EBNA3A, 3B and 3C family of proteins.

Interestingly, the size or the response to an epitope observed in primary infection does not correlate with memory responses to an epitope of the same type (Hislop *et al.*, 2002) and clones that dominate in the acute infection are heavily culled as the primary response decreases and T-cell memory arises (Callan *et al.*, 2000). In a study by Catalina and colleagues, they show that whille specific $CD8^+$ T cells to the lytic protein BMLF-1 are maintained through latency, $CD8^+$ T cell specificities to other lytic proteins, BZLF-1 and BRLF-1, were not maintained. On the other hand, specificities to latent antigens were maintained 1 year post EBV infection (Catalina *et al.*, 2001). In another study, BZLF1-specific $CD8^+$ T cells continuously increased during the presentation of IM and were still present during convalescence (Precopio *et al.*, 2003).

Even though latent EBV infection is usually largely controlled, viral shedding in the oropharynx remains high for months in patients with IM. Upon observing responses localized to the tonsils, it was shown that during acute IM, EBV-specific effectors were much less frequent in the tonsil than in blood. In recently recovered patients, $CD8^+$ T cells specific to latent epitopes were faster to acquire CCR7, populate the tonsil and remain there, relative to lytic specificities. On the other hand, asymptomatic EBV carriers not only possessed a large proportion of total tonsillar EBV-specific $CD8^+$ T cells, but also a great enrichment of both lytic

and latent epitope reactivities in the tonsil, suggesting appropriate homing of EBV-specific cells during efficient immune control (Hislop *et al.*, 2005).

EBV-specific $CD8^+$ T cells in IM blood are of $CD45RO^+$ activated effector phenotype (Callan *et al.*, 1998). They additionally express intracellular perforin, are cytotoxic and show heterogeneity in inflammatory cytokine production. As the majority of T cells are culled following acute infection, EBV-specific T cells are sensitive to apoptosis. It has been demonstrated that the majority of EBV-specific T cells in IM patients have low levels of anti-apoptotic Bcl-2 (Tamaru *et al.*, 1993; Callan *et al.*, 2000) and are programmed for cell death via the mitochondrial/cytokine rescuable pathway. When PBMCs isolated from IM patients were stimulated with cytokines IL-2, IL-7 and IL-15, it improved the survival of EBV-specific T cells, protecting them against cell death (Callan *et al.*, 2000).

EBV-specific memory $CD8^+$ T cells specific for lytic and latent epitopes are negative for activation markers, yet still keep their cytotoxic potential, as seen in *ex vivo* studies (Hislop *et al.*, 2001). Even though much lower than in acute disease, lytic epitope-specific $CD8^+$ T cells constitute around 0.2% to 2% and latent specificities 0.05% to 1% of the total $CD8^+$ T cell population to control the asymptomatic infection (Hislop *et al.*, 2007). At IM diagnosis, EBV-specific $CD8^+$ T cells consist mainly of $CD27^+$ memory T cells and $CD27^-$ effector T cells. Six months after IM diagnosis, the remaining $CD8^+$ T cells consist mainly of the $CD27^+$ memory T cells (Scherrenburg *et al.*, 2008).

Unlike for $CD8^+$ T cells, much is unknown about the response of $CD4^+$ T cells in primary EBV infection, mostly due to their low frequency. $CD4^+$ T cells did not expand during early stages of acute EBV infection, however it could be possible that a slight expansion occurred during the incubation time before disease onset and patient recruitment. During IM, up to 2.7% of circulating effector and memory $CD4^+$ T cells are EBV specific. In contrast with the $CD8^+$ T cell response largely recognizing lytic proteins, the $CD4^+$ cell response is directed against both lytic and latent antigens (Precopio *et al.*, 2003). $CD4^+$ T cells against an epitope derived from the lytic antigen BHRF1 were identified. These T cell clones showed cytolytic action against autologous and allogeneic LCLs, together with strong IFNγ production, suggesting a contribution of EBV-specific $CD4^+$ T cells in the control of EBV replication (Landais *et al.*, 2004). During IM, $CD4^+$ T cells specific for the lytic antigen BZLF1 were highest at presentation and dominant over $CD8^+$ T cells, and declined 6 months after IM (Precopio *et al.*, 2003; Scherrenburg *et al.*, 2008). On the other hand, $CD4^+$ and $CD8^+$ T cells specific for EBNA1 were low for half a year following IM (Scherrenburg *et al.*, 2008).

During latency, memory EBV-specific $CD4^+$ T cells are likely present in low numbers and may be kept at sites of viral reactivation such as the tonsil. In a study by Paludan and

colleagues, EBNA1-specific $CD4^+$ T cells in the peripheral blood of healthy individuals with latent EBV infection represent around 0.5% of $CD4^+$ T cells (Paludan *et al.*, 2002). EBV-specific $CD4^+$ T cell lines generated from healthy, EBV-seropositive individuals were specific for EBNA1 and EBNA3C (Munz *et al.*, 2000; Steigerwald-Mullen *et al.*, 2000) and specificities to EBNA1, EBNA3C, LMP1 and LMP2 were detected with ELISPOT assays (Leen *et al.*, 2001).

Interestingly, stimulating donor-derived T cells with autologous LCLs *in vitro* generates EBV-specific T cells that have been used successfully to target EBV positive malignancies, such as EBV positive post-transplant lymphoproliferative disease (PTLD) (Linnerbauer *et al.*, 2014). The $CD4^+$ T cells in these cultures express IFNγ and cytotoxic molecules and can control outgrowth of tumor cells *in vitro* (Long *et al.*, 2009) and in an *in vivo* mouse model of PTLD (Linnerbauer *et al.*, 2014). The specificities of the $CD4^+$ T cells in these cultures are largely unknown. $CD4^+$ T cells specific for virion antigens were tumor-protective, however the best effectors were $CD4^+$ T cells likely specific for self-antigens upregulated by transformed cells (Long *et al.*, 2009; Linnerbauer *et al.*, 2014).

1.1.6 Humoral immunity to EBV

Primary acute infection can be diagnosed serologically by the presence of IgM antibodies against the viral capsid antigen (VCA) in the absence of IgG to EBNA1. During transition from acute primary to persistent infection, VCA IgM antibodies disappear and VCA and/or EBNA1 IgG antibodies emerge (Silins *et al.*, 2001; Middeldorp, 2015).

In summary, EBV is a ubiquitous human herpesvirus which can cause acute infectious mononucleosis in a portion of individuals. In cases of weaker EBV immune surveillance such as during immunosuppression, EBV is associated with several cancers (Delecluse *et al.*, 2007). On the other hand, a heightened and dysregulated response to primary EBV infection is associated with autoimmunity, particularly multiple sclerosis (Ascherio and Munger, 2015).

1.2 Multiple Sclerosis

1.2.1 Pathology

Multiple sclerosis (MS) is an immune-mediated, demyelinating and neurodegenerative disease of the central nervous system (CNS) with multifactorial etiology. The pathological hallmark of the disease is the formation of demyelinating lesions found in the grey and white matter of the brain and spinal cord, yet the clinical manifestations of the disease are heterogeneous (Sospedra and Martin, 2005). As a major cause of neurological disability in young adults, symptoms include changes in vision, sensation, mobility, cognition and balance (Brownlee *et al.*, 2017).

Four clinical courses of MS have been defined (Lublin and Reingold, 1996). The diagnosis is largely based on the presence of symptoms and MRI findings of CNS lesions characteristic to MS (Polman *et al.*, 2011); their dissemination across the CNS and dissemination over time (Filippi *et al.*, 2018). The majority of patients (85-90%) experience episodes of reversible neurological dysfunction, or relapses, in the initial phases of the disease, known as the clinically isolated syndrome (CIS) and during relapsing-remitting MS (RRMS) (Fig. 2). These relapses last several days to weeks, occur at irregular intervals with varying neurological recovery and are followed by periods of remission. The mean age at onset of RRMS is 30 years, however there are cases of pediatric MS, where the first demyelination event takes place before the age of 18 (Yeshokumar *et al.*, 2017). The disease is more prevalent in women, with female to male ratio of around 3:1 (Orton *et al.*, 2006). With time, the disability may progress, permanent neurological deficits can develop and the disease is then characterized as secondary progressive MS (SPMS) (Lublin and Reingold, 1996).

A minority of patients (10-15%) is diagnosed with primary progressive MS (PPMS), in which permanent neurological disability progressively increases from disease onset (Polman *et al.*, 2011). The mean age at onset is higher than for RRMS (40 years) and without a sex bias (Lublin *et al.*, 2014). Finally, progressive relapsing MS (PRMS) is a rare disease course that is characterized by the continuing progression of disease from onset with periods of acute relapses (Lublin and Reingold, 1996). In addition, the subtypes of the disease can be classified as active or inactive depending on clinical observations, relapses and lesion activity (Lublin *et al.*, 2014).

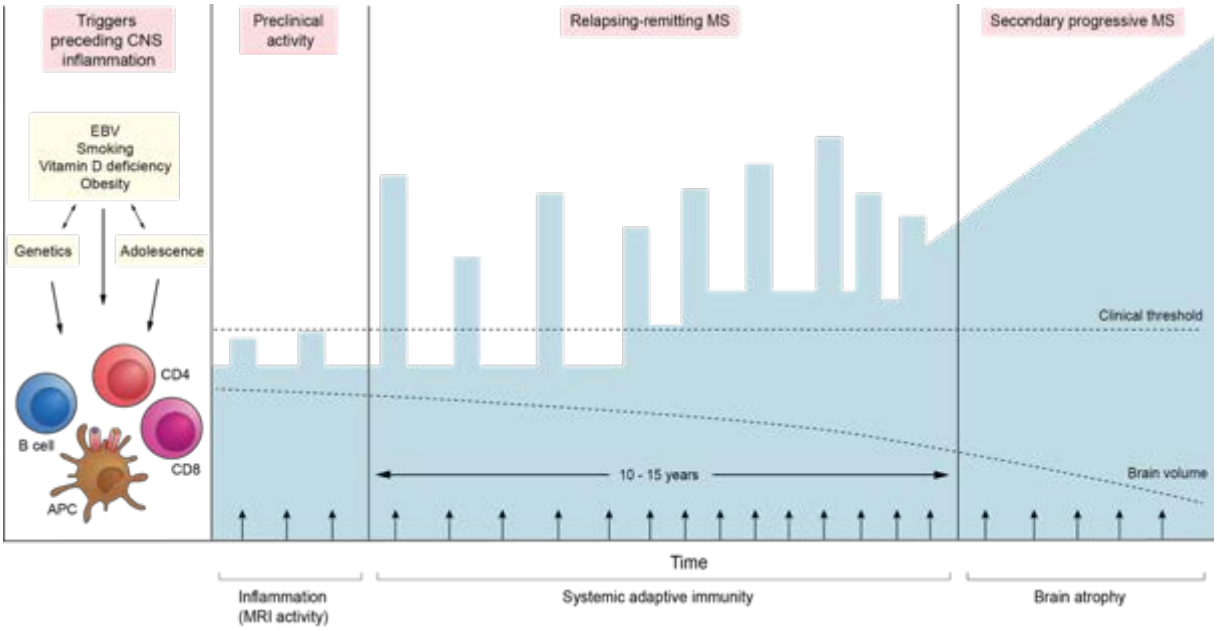

Figure 2: Evolution and disease course of MS. During the subclinical phase before symptom onset, many risk factors interact together to predispose to MS, including genetics, EBV infection, smoking, vitamin D deficiency and obesity. The important time for these interactions is adolescence. Blue area represents disease progression, with spikes indicating damage to the CNS during RRMS, followed by a steady increase in neurological dysfunction during SPMS. During the course of disease, brain volume decreases and inflammatory episodes in the CNS are more frequent than clinical relapses (black arrows).

(Adapted from (Olsson *et al.*, 2017).

1.2.2 MS immunopathology

1.2.2.1 Infiltrating lymphocytes

In 1868, Charcot was the first to describe MS symptoms and lesions in the brain and spinal cord (Pearce, 2005). Inflammatory lesions contain different types of immune cells. Most are T cells, dominated by $CD8^+$ T cells, and they infiltrate early in lesion formation (Dendrou *et al.*, 2015). It was demonstrated that the majority of infiltrating $CD8^+$ T cells in actively demyelinating lesions belonged to a few clones, while there was more heterogeneity amongst $CD4^+$ T cells (Babbe *et al.*, 2000). These active lesions are mostly observed in patients with acute or RRMS. In contrast to active lesions, inactive lesions contain lower numbers of T cells (Frischer *et al.*, 2009). MS is believed to be an autoimmune disease with autoreactive lymphocytes initiating responses against CNS autoantigens, the nature of which is unknown (Dendrou *et al.*, 2015). The disease is thought to be induced and perpetuated by autoreactive $CD4^+$ T cells for the following reasons. $CD4^+$ T cells infiltrate into the CNS of MS patients and

the main genetic risk for MS is conferred by major histocompatibility complex (MHC) class II molecules, HLA-DR and DQ. Additionally, humanized mice transgenic for these molecules and/or myelin-basic protein (MBP)-specific T cell receptors are susceptible to experimental autoimmune encephalomyelitis (EAE) and finally, $CD8^+$ T cell maturation, antibody production, inflammatory cytokine production and other functions are partly controlled by $CD4^+$ T cells (Sospedra and Martin, 2005) (Fig. 3).

B cells are also present, but on average the numbers are 10 times lower than those of T cells. Plasma cells are only sparsely present and show little relation to lesional activity (Frischer *et al.*, 2009).

Most profound inflammation is observed in patients with acute or RRMS, followed by progressive disease. Interestingly, in late stages of disease, inflammation is seen to considerably decline in a large proportion of patients. Even though T and, to a lesser extent, B cells are markers for disease activity and tissue damage, they interestingly show different distribution patterns. While T cells are seen especially in active lesions and CNS parenchyma, B cells and plasma cells tend to accumulate in the connective tissue spaces of the brain, like the meninges and perivascular spaces. In addition, plasma cells accumulate in later disease stages and can persist even after T and B cell inflammation is cleared (Frischer *et al.*, 2009), which could explain long-lasting oligoclonal bands in the CSF (Meinl *et al.*, 2006).

Highlighting the role of B cells in MS, B cell-depleting antibody treatments, such as rituximab (anti-CD20) and ocrelizumab (anti-CD20) have shown positive effects in MS patients through the reduction of inflammatory brain lesions (Hauser *et al.*, 2008). An important question in MS is how disease-relevant T and B cells interact with each other before disease onset in the periphery and also in structures such as the tertiary lymphoid structures in the meninges of MS patients. Indeed, B cells can contribute to MS pathogenesis by acting as antigen-presenting cells (APCs) to autoreactive T cells and providing co-stimulatory signals, recruiting autoreactive T cells into the CNS and producing myelin-specific antibodies which can lead to myelin destruction (Sospedra and Martin, 2005). Interestingly, auto-proliferating peripheral T helper 1 (T_H1) cells are more frequent in MS patients positive for the HLA-DRB1*15:01 (HLA-DR15) haplotype, which is mediated by memory B cells in an HLA-DR-dependent manner. Depletion of B cells with anti-CD20 reduces this autoproliferation (Mohme *et al.*, 2013; Jelcic *et al.*, 2018).

Therapies that target inflammation and the immune system have been effective in RRMS, but with the exception of the B cell depleting therapies not in progressive MS, pointing towards different mechanisms being responsible for these disease stages (Lassmann, 2013) and for driving relapses versus chronic disease progression. In the early stages of MS, the

inflammation is associated with a leaky blood-brain barrier (BBB), allowing inflammatory cells to enter the CNS. On the other hand, in progressive disease, signs of inflammation are rather seen around vessels with an intact BBB (Hochmeister *et al.*, 2006), suggesting that the inflammatory response could be partly trapped in the CNS. Indeed, in patients with SPMS, inflammatory cell-containing structures resembling lymphoid follicles with high numbers of B cells and plasma cells were observed in the meninges (Serafini *et al.*, 2004). These structures were associated with an increase in meningeal inflammation, grey matter cortical demyelination and accelerated clinical course (Howell *et al.*, 2011). However, secondary progressive disease might not only be the follow-up after relapsing-remitting phase, but might in fact be the result of a distinct pathophysiological mechanism (Dendrou *et al.*, 2015).

Experimental autoimmune encephalomyelitis (EAE) is the accepted animal model of MS, in which CNS inflammation and disability are results of sensitization with CNS antigens. While EAE has provided valuable insights into basic immune mechanisms in the CNS, there have also been some discrepancies with human MS (Gold *et al.*, 2006). Several therapies which seemed attractive from EAE studies failed in human patients.

EAE worsened under TNF treatment (Kuroda and Shimamoto, 1991) and there was disease protection after administration of neutralizing anti-TNF antibodies (Ruddle *et al.*, 1990). However, when lenercept, a recombinant TNF receptor, was used in a phase II clinical trial, patients experienced exacerbations of disease and the exacerbations occurred earlier when compared with patients receiving placebo. Neurologic deficits in addition were more severe in lenercept-treated patients (1999). IFNγ was partially protective in EAE, however administering it to patients resulted in disease exacerbation and relapses (Panitch *et al.*, 1987). Similarly, the interleukin (IL)-12 p40 cytokines IL-12 and IL-23, with central roles in the differentiation of the proinflammatory T_H1 and T_H17 $CD4^+$ T cells, have been implicated in both MS and EAE. Neutralizing the IL-12 p40 subunit prevented EAE development in rodents and non-human primates (Leonard *et al.*, 1995; Brok *et al.*, 2002) and in marmosets was able to delay demyelination and suppress inflammation of pre-existing brain lesions (t Hart *et al.*, 2005). However, when MS patients were treated with ustekinumab, a neutralizing human monoclonal antibody against IL-12/23 p40, their disease activity was not improved (Segal *et al.*, 2008), suggesting either poor availability to the CNS, or the involvement of other proinflammatory pathways in MS pathogenesis other than T_H1 and T_H17 (Martin, 2008).

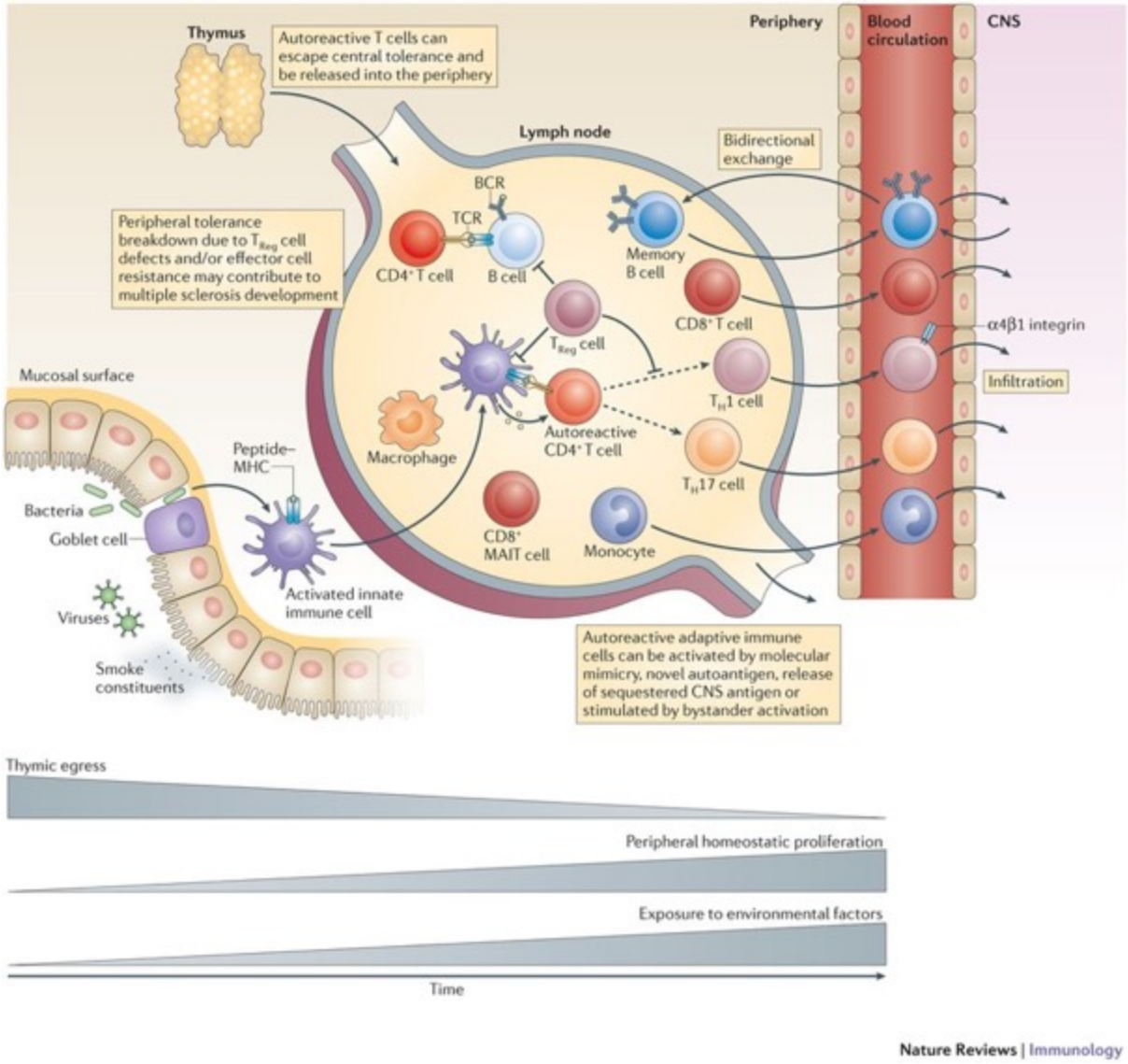

Figure 3: Peripheral immune system dysregulation. Most autoreactive T cells are deleted in the thymus as part of central tolerance. Nevertheless, some autoreactive T cells are released into the periphery and should be removed during peripheral tolerance. If this process is defective, either by inefficient function of regulatory T cells (T_{REG}) or resistance of lymphocytes, autoreactive T and B cells can get activated, differentiate into effector cells through processes of molecular mimicry, novel autoantigen presentation, bystander activation or leak of CNS antigens into the periphery. Differentiated activated lymphocytes can then infiltrate into the CNS and trigger or perpetuate inflammation and tissue damage. Both environmental and genetic risk factors act alone or interact together to contribute to these events. Dashed arrows indicate differentiation.

(Dendrou *et al.*, 2015).

1.2.2.2 CNS-resident cells

Macrophages and microglial cells were observed in all active lesions (Frischer *et al.*, 2009). The highest numbers of macrophages infiltrate early and late active lesions, typically seen in acute or RRMS, and contain different myelin components in their lysosomes (Bruck *et al.*, 1995). On the other hand, the majority of lesions in progressive disease show an inactive lesion core, surrounded by a thin edge of activated microglia and macrophages (Prineas *et al.*, 2001).

Oxidative burst and oxidative damage, mediated by activated macrophages and microglia driven by inflammation, is an important mechanism contributing to tissue injury in all lesions in MS (Lassmann and van Horssen, 2011). Oxidative damage causes mitochondrial injury and dysfunction. This can mediate tissue damage through processes of energy failure, apoptosis induction and increased production of reactive oxygen species (ROS), as well as being involved in oligodendrocyte destruction (Veto *et al.*, 2010). Indeed, the molecules that play a role in oxidative burst pathways are strongly up-regulated in active MS lesions (Lassmann and van Horssen, 2011).

Damage to oligodendrocytes, in addition to their destruction through apoptosis (Barnett and Prineas, 2004) and consequent demyelination, are results of inflammation. Axonal degeneration, likely caused by the effects of the inflammatory environment, is a consequence of demyelination and their damage is seen in both active and chronic lesions in the brains of MS patients (Trapp *et al.*, 1998). Acute axonal damage is most extensive in the early stages of disease and correlates to the numbers of $CD8^+$ T cells (Kuhlmann *et al.*, 2002). In addition, with lesion maturation, axonal loss is seen and correlates to irreversible neurological disability (Bjartmar *et al.*, 2000). Upon examining all lesion stages from MS patients, a correlation is observed between the number of inflammatory cells with the extent of acute axonal injury. The correlation is strongest for T cells, HLA-D positive macrophages and microglia and was observed in all MS patients (Frischer *et al.*, 2009).

1.2.3 Genetic MS risk factors

MS is a multifactorial autoimmune disease and the etiology of the disease is not yet completely understood. The disease clusters with complex genetic diseases, characterized by moderate disease-risk heritability and intricate gene-environment interactions (Oksenberg *et al.*, 2008). There are hundreds of risk factors that have been implicated in this disease, both genetic and environmental.

1.2.3.1 HLA locus

MHC molecules present extracellular or intracellular peptides to T cells and are essential for the start of the adaptive immune response. As is the case with many autoimmune diseases, the human leukocyte antigen (HLA) gene cluster on chromosome 6p21.3 coding for MHC has for decades been associated with the disease. Now, with clear evidence, it is the strongest susceptibility locus for MS in the genome (Sawcer *et al.*, 2005). From this HLA cluster, the MHC class II haplotype, which consists of the alleles HLA-DRB1*15:01 (DR2b, or HLA-DR15), HLA-DRB5*01:01 (DR2a), HLA-DQA1*01:02 and HLA-DQB1*06:02 existing in linkage disequilibrium, bears the greatest effect on risk (Sawcer *et al.*, 2014). Isolating the association of individual variants has been difficult due to the extensive polymorphism and linkage disequilibrium that exists within the MHC region of the genome (Horton *et al.*, 2008). In the last few years however, with the rise of technical and statistical methods to type for single nucleotide polymorphisms (SNPs) in large cohorts, progress has been made in identifying further genetic determinants (Sawcer *et al.*, 2005; Bush *et al.*, 2010). It has become apparent that the HLA-DR15 allele drives the association, with the other alleles in the haplotype being associated due to linkage disequilibrium with HLA-DR15 (Patsopoulos *et al.*, 2013).

The MHC class II variant HLA-DR15 increases the risk of MS with an odds ratio (OR) of 3, whilst an MHC class I variant HLA-A*02 associates with protection from the disease (OR ~ 0.6) (Brynedal *et al.*, 2007; Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Patsopoulos *et al.*, 2013).

1.2.3.2 Non-HLA loci

Over the last decade, several genome-wide association studies (GWAS) identified many loci that appeared to have a role in MS susceptibility (Hafler *et al.*, 2007; Wellcome Trust Case Control *et al.*, 2007; Australia and New Zealand Multiple Sclerosis Genetics, 2009; Baranzini *et al.*, 2009; De Jager *et al.*, 2009; Sanna *et al.*, 2010; Patsopoulos *et al.*, 2011). However, it seemed larger sample sizes would be required to examine the complex genetic architecture. In 2011, the International Multiple Sclerosis Genetics Consortium (IMSGC) performed a collaborative GWAS, including 9777 subjects. In addition to replicating the majority of the loci previously observed, this work identified 29 novel variants associated with MS risk (Sawcer *et al.*, 2011). Two years later, the IMSGC expanded this to include five additional loci, which in the previous study have shown sub-genome-wide significance (International Multiple Sclerosis Genetics *et al.*, 2013) and also performed an ImmunoChip custom genotyping array on 14 498 multiple sclerosis subjects, identifying further 48 new

susceptibility loci (Beecham *et al.*, 2013). In the last few years, two larger studies from the IMSGC genotyping genome-wide data of tens of thousands of MS patients and controls increased the number of risk variants associated with MS to >230, mostly lying outside the MHC locus (Cotsapas and Genetics, 2018; International Multiple Sclerosis Genetics, 2019).

In the first described 110 variants, 15 of them were predicted to be coding variants, with an additional 35 in strong linkage disequilibrium with other coding variants. Also, all of these SNPs coincide with areas of chromatin suggestive of regulatory function; 91 variants change at least one regulatory sequence motif and a further 18 are in linkage disequilibrium with other variants that can do so. Moreover, 27 variants are placed within transcription-factor binding sites and again, 51 show to be in linkage disequilibrium with similar SNPs. Among the variants, 15 are quantitative trait loci for gene expression, with 16 in linkage disequilibrium with others (Sawcer *et al.*, 2014). The nearest gene to the specific SNP was identified and interestingly, these MS-associated SNPs frequently lie in gene regulatory regions of genes that mainly play a role in the adaptive immune response, more specifically in T cell activation and proliferation and T helper cell differentiation. The relevant genes include cytokine pathways, co-stimulatory molecules and immunologically-relevant signal transduction molecules (Sawcer *et al.*, 2014). In a larger meta-analysis by the IMSGC in 2018, low-frequency risk variants were shown to make up 5% of MS heritability. Whilst the study also highlights T cell homeostasis pathways, IFNγ biology and NFκB signaling in MS pathogenesis, it identifies four novel genes driving MS risk, two of which (PRKRA and NLRP8) are involved in innate immunity and HDAC7 with roles in thymic T cell development (Cotsapas and Genetics, 2018).

Interestingly, the study by IMSGC in 2019 mapping MS genomics highlights pathways involved in both innate and adaptive immunity and spanning many cell types, with an enrichment of MS-associated genes was seen in microglia. In addition, a few loci influence gene expression in the human cortex but not in individual immune cells within and there is robust evidence for a susceptibility locus on the X chromosome (International Multiple Sclerosis Genetics, 2019). All these studies further highlight the immune-mediated pathogenesis of MS (Sawcer *et al.*, 2014).

The direct effects and roles of these risk variants are largely unknown, however a few have observable functional effects. As one example, the SNP rs1800693 in *TNFRSF1A* leads to higher concentrations of the soluble form of the tumor necrosis factor receptor 1 (TNFR1), which can in turn block TNF (Gregory *et al.*, 2012). As drugs that act to block TNF have led to exacerbation of MS (van Oosten *et al.*, 1996; Arnason *et al.*, 1999), this work clearly shows the importance of studying the functional consequences of the disease-association risk variants.

The MS-associated variants that have been discovered are thought to only represent about 25% of the heritability in the disease. While another 25% could account for variants that have not yet been discovered, it is possible that the other 50% is "phantom heritability" (Zuk *et al.*, 2012), which results from risk factor interactions (Sawcer *et al.*, 2014). Even though the odds ratios of individual SNPs are estimated to be quite small (around 1.14-1.58), it is very unlikely that they act in isolation, as the genetic risk of MS is clearly polygenic (Bush *et al.*, 2010).

Within families, the risk of MS between siblings was reported to be 15-20-fold increased, and the concordance rate between identical twins was 30% (Oksenberg *et al.*, 2008; Beecham *et al.*, 2013). However, more recent population-based studies have estimated the sibling's risk to be 7-fold (Westerlind *et al.*, 2014). Together, this suggests that a large portion of MS risk lies outside genetics.

1.2.4 Environmental MS risk factors

Even though MS is a relatively common disease, the burden of MS across the world is not the same. There are a great number of environmental factors that influence MS risk, ranging from gender, age, ethnicity, latitude to viral agents. MS is a rare disease in the tropical and subtropical regions and the incidence and prevalence increases with latitude, both to the north and south of the equator. The prevalence saw a 10.4% increase from 1990 to 2016, with highest to lowest prevalence estimated in high-income North America (164.6 per 100 000 population), western Europe (127.0), Australasia (91.1), eastern sub-Saharan Africa (3.3), central sub-Saharan Africa (2.8) and Oceania (2.0) (Wallin *et al.*, 2019).

Among pre-teen children, the prevalence of MS is similar between the two sexes, however during adolescence, the prevalence starts to diverge, increasing more for girls than for boys (Wallin *et al.*, 2019). Women additionally see a higher increase in MS incidence (Koch-Henriksen *et al.*, 2018), and the female to male ratio is 1.5-2.5:1 in most populations (3.2:1 in Canada) and increasing over the last 50 years (Orton *et al.*, 2006). As genetic differences are unlikely in this short period of time, the increase is likely to result from environmental or gene-environment interactions (Orton *et al.*, 2006).

Whilst genetic predisposition is a very likely contributor to the geographic variations, migration studies further support a role for environmental factors. A crucial period of exposure to environmental triggers seems to be in the first two decades of life, as immigrant children take on the MS risk of the host country, whilst older migrants retain the low risk of their country of origin. Likewise, migrants that have come from low risk countries to high risk retain low risk, however their children show an MS risk approaching the host country (Gale and Martyn, 1995).

The possibility of an infectious agent responsible for the epidemiology of MS was examined. Two hypotheses were originally proposed. The first, termed the *poliomyelitis hypothesis* (Poskanzer *et al.*, 1963), postulated that a virus acquired early in childhood would confer protection, whereas if acquired later in life would increase the risk of MS. The second, termed *prevalence hypothesis* (Kurtzke, 1993), suggested that a high-risk MS area has a common pathogen, which asymptomatically persists in the majority of individuals and only rarely, and years after primary infection, could cause MS symptoms. With more evidence for the former hypothesis, it changed more into a general *hygiene hypothesis*. First proposed in a study in Israel, it linked better hygiene to the etiology of MS (Leibowitz *et al.*, 1966) and suggested that the increased prevalence of autoimmune diseases in industrialized countries is partly due to the reduction of infectious diseases in these areas (Bach, 2002). Additionally, hypotheses arose that the pathogenesis of MS could be autoimmune, with a common pathogen driving or initiating autoimmunity, and the risk increasing with age of infection (Hafler, 1999; Hunter and Hafler, 2000). Due to a large array of infectious agents and diversity of immune responses, the chance of all infectious agents to be involved in MS risk was very unlikely. Interestingly, EBV became a pathogen of interest (Fraser *et al.*, 1979; Warner and Carp, 1981).

1.2.4.1 EBV

As EBV infects the majority of the worldwide population, the associated increase in MS risk has been puzzling for many years. However, studies in pediatric MS patients helped to strengthen the evidence. In a pediatric MS cohort, it was demonstrated that 83% of patients had serological evidence of EBV infection, compared to 42% control children (Alotaibi *et al.*, 2004), and almost complete EBV seropositivity (98.6% pediatric MS patients versus 72.1% in controls) in a larger study (Pohl *et al.*, 2006). Additionally, utilizing the stored serum of >8 million active-duty personnel, Levin and colleagues could show that all adult MS patients seroconverted to EBV before their disease onset. This argues that EBV seronegative individuals have an extremely low risk of MS, but sharply increase their risk following EBV seroconversion (Levin *et al.*, 2010). Asymptomatic EBV infection confers MS risk, however this risk sharply increases following IM in adolescence and early adulthood (Thacker *et al.*, 2006; Handel *et al.*, 2010; Endriz *et al.*, 2017). Interestingly, this risk synergizes with the HLA-DR15 haplotype for a 7-fold increased MS risk (Olsson *et al.*, 2017). An important question in MS is how does EBV infection contribute to the risk of MS, whether it is a question of cross-reactivity and molecular mimicry and/or a general role in immune dysregulation, and the mechanisms behind the synergy of genetics with EBV. These and related questions on how an altered immune response to EBV contributes to MS risk will be discussed in more details in this thesis.

1.2.4.2 Smoking, vitamin D deficiency and obesity

Several other environmental risk factors have been linked to MS. Smokers of both sexes have an increased risk of developing MS (OR 1.5) and the increase in smoking dose increases the risk (Hedstrom *et al.*, 2009; Handel *et al.*, 2011). In addition, smoking interacts with MS-associated HLA risk alleles, further increasing the ORs. As even passive smoking shows an increased risk (OR 1.3), the effects might be a result of nonspecific lung irritation (Hedstrom *et al.*, 2011; Hedstrom *et al.*, 2014a), together with smoking-protomoted proinflammatory pathways (Shan *et al.*, 2009) and citrunillation of peptides (Klareskog *et al.*, 2009).

Low vitamin D levels also increase MS risk. In a study by Munger and colleagues using stored serum of US military personnel, increasing levels of vitamin D significantly decreased the risk of MS in Caucasians, especially before 20 years of age (Munger *et al.*, 2006). Suggesting an important role in T cell homeostasis, vitamin D was able to inhibit the proliferation of $CD4^+$ T cells isolated from MS patients. In addition, a boost in the development of IL-10-producing cells and an increase in regulatory T cells was seen, whilst reducing the amounts of cells secreting IL-6 and IL-17 (Correale *et al.*, 2009). Interestingly, an interaction between vitamin D and the HLA-DR15 allele has also been observed in *in vitro* studies. A highly conserved vitamin D response element was found in the promoter of this specific HLA allele and was responsive to vitamin D in influencing gene expression. This responsiveness was not observed with other HLA-DRB1 haplotypes (Ramagopalan *et al.*, 2009). Even though this finding was not reproduced in a case-control study (Baarnhielm *et al.*, 2012), the interaction is yet to be understood.

Finally, adolescent obesity has been associated with an increased risk for MS (Munger *et al.*, 2009; Hedstrom *et al.*, 2012) and particularly in girls for pediatric-onset MS (Langer-Gould *et al.*, 2013). Again, an interaction with HLA-DR15 has been observed (Hedstrom *et al.*, 2014b). While the mechanisms behind the interactions are unknown, it is hypothesized that chronic, obesity-related inflammation and activation of the innate immune system (Gregor and Hotamisligil, 2011) could lead to a boost in the activation of autoreactive T cells via HLA presentation (Lumeng *et al.*, 2007; Hedstrom *et al.*, 2014b). As obese people have lower levels of vitamin D, another hypothesis points towards the effect of lower levels of this metabolite (Wortsman *et al.*, 2000). Interestingly, an interaction has been observed between obesity and EBV infection (Hedstrom *et al.*, 2014b) and remains largely speculative.

In summary, MS is an autoimmune disease believed to be induced and perpetuated by autoreactive $CD4^+$ T cells. Whilst more than 200 genetic risk factors and several environmental risk factors have been associated with MS risk, their exact roles in disease pathomechanisms

still require further study. In particular, the interactions between MS genetics and the environment, mainly EBV infection, remain to be understood and were studied in this thesis.

1.3 Humanized mice

1.3.1 Humanized mouse models

The study of EBV infection in a murine model is difficult, as the gamma 1 herpesvirus subfamily developed in and evolved with monkeys and primates (Ehlers *et al.*, 2010) and thus has a narrow species tropism. The murine gamma-herpesvirus 68 is a close relative to EBV and shares some similarities making it a valuable small animal model. However, differences such as the lungs as the site of acute infection, infection of not only B cells but also macrophages, dendritic cells and lung epithelial cells, low B cell transforming capacity and specificity of the $CD8^+$ T cell expansion (Flano *et al.*, 2002), amongst others, has made it a less ideal suitable candidate to study acute EBV infection and tumorigenesis.

As a model to investigate the basic biology of EBV, as well as gain an insight into EBV-specific immune control, mice reconstituted with human immune system components have shown success. These are severely immunodeficient mouse strains, which allow for the engraftment of human cells and study of human-tropic pathogens. The most popular are the BALB/c or non-obese diabetic (NOD) mouse strains, which harbor *scid* or RAG mutations together with deletions of the common gamma chain (γ_c) of interleukin (IL)-2,4,7,9,15 and 21 (BALB/c $RAG2^{-/-}$ $\gamma_c^{-/-}$: BRG and NOD-*scid* $\gamma_c^{-/-}$: NSG). With these mutations, animals lack mature murine T and B lymphocytes, as these cells cannot somatically rearrange or repair their receptors. In addition, without signaling through the γ_c, the development of murine innate lymphocytes is abolished, as well as IL-15-dependent NK cell maturation (Ito *et al.*, 2002; Traggiai *et al.*, 2004; Ishikawa *et al.*, 2005; Shultz *et al.*, 2005).

NSG animals injected with $CD34^+$ progenitor cells reconstitute up to 50-80% of mature human blood cells, including myeloid cells, dendritic cells and lymphocytes. Human cells are found in peripheral blood, bone marrow, spleen and thymus. The thymus displays some signs of a typical thymic architecture with cortex and medulla structures with mostly $CD3^+$ human T cells, as well as human B cells and human DC-like APCs (Yahata *et al.*, 2002). In addition, the human $CD3^+$ thymocytes express TCR-αβ, suggestive of terminally differentiated thymocytes and hence evidence for a thymic program capable of leading full T cell development. In the periphery, the majority of T cells express either CD4 or CD8, TCR-αβ and CD45RA, suggestive of a naïve T cell phenotype. A fraction of T cells also express TCR-γδ (Yahata *et al.*, 2002). Reconstitution is maintained for up to a year and unlike with the earlier NOD-scid animal model, T cell lymphomas were not observed (Ishikawa *et al.*, 2005).

In a NOD/shi-scid/γ_c^{null} (NOG) humanized mouse model reconstituted with cord blood-derived $CD34^+$ hematopoietic stem cells, most of the IgM and/or IgG positive B cells are in

peripheral blood in the naïve state. Around 40% of B cells in the spleen are B cell progenitors. In *in vitro* cultures, stimulated IgD^+ B cells were able to produce both IgM and IgG, suggesting responsiveness of mature B cells to antigenic challenge and Ig class switch capacity. Development of both $CD4^+$ and $CD8^+$ T cells was observed in both the thymus and spleen and while they proliferated upon stimulation, the response was not robust, especially from $CD4^+$ T cells. Upon examining $CD4^+$ T cells isolated from the thymus or spleen, this unresponsiveness was induced in the periphery as thymic T cells showed strong responses, suggesting mechanisms of tolerance induction against mouse periphery. Interestingly, a small percentage of human $CD4^+$ T cells reconstituted with a significant delay in a NOG mouse model lacking I-A, suggesting the HLA molecules on the human T cells, B cells or dendritic cells were able to positively select T cells in the thymus. Nevertheless, the delay in T cell development suggests that I-A on mouse thymus epithelial cells plays a significant role in the human T cell positive selection (Watanabe *et al.*, 2009).

1.3.2 T cell selection in humanized mice

How human T cells are selected on mouse thymic cells remains unclear. However, it has been shown that in the BRG and NSG model, reconstituted T cells were able to discriminate between self and allogeneic MHC (Yahata *et al.*, 2002; Traggiai *et al.*, 2004; Ishikawa *et al.*, 2005). In the NSG mouse, the thymus is mostly populated with human T cells and a few B cells. Most of these T cells are immature $CD4^+CD8^+$ double positive, with a small fraction of single positive cells, suggesting the presence of T cell development in the mouse thymus (Ishikawa *et al.*, 2005). As the development of T cells is restricted by human MHC (hMHC), it is possible that human B cells and DCs play a role in the thymocyte selection (Yahata *et al.*, 2002). As another possibility, mouse epithelial cells might also support the education of human T cells (Yahata *et al.*, 2002), as the human CD8 co-receptor is able to interact with murine MHC (mMHC) class I (Moots *et al.*, 1992) and human CD4 with mMHC class II (Vignali *et al.*, 1992). Indeed, MHC class II positive mouse thymic microenvironment was able to positively select TCR-$\alpha\beta^+$ $CD4^+$ single positive human cells. However, these cells were not responsive to PHA and IL-2 and did not represent a developmentally end stage population. While they are able to interact with mMHC class II during maturation, a species-specific signal is then required for full maturity and functionality (Res *et al.*, 1997). Reconstituted T cells did not respond to murine cells, indicative of tolerance. In addition, these cells are also not reactive to autologous HLA human antigens, suggesting additional tolerance to autologous antigens, possibly presented by human cells in the thymus. As a result, during negative selection in the mouse thymus, it is possible that cells reactive to both autologous human and murine antigens have been deleted (Yahata *et al.*, 2002).

1.3.3 EBV infection in humanized mice

One method of humanized mouse generation, and a method that is employed in our laboratory, includes the intrahepatic injection of human fetal liver-derived hematopoietic progenitor cells (HPCs) into sublethally irradiated, newborn mice. Following EBV infection of these animals with the B95-8 EBV strain, which was isolated from an American patient with IM (Miller and Lipman, 1973), we see specific viral immune control. IFN-α responses by plasmacytoid dendritic cells are seen early in infection, followed by NK cell responses to lytic EBV antigens between weeks 2 and 5 of the infection, and a protective mainly $CD8^+$ T cell expansion starting to be prominent from week 4 (Chatterjee *et al.*, 2014; Gujer *et al.*, 2015). Splenomegaly observed in these animals is likely due to T cell expansion in responses to increasing viral titers.

EBV infection in humanized mice can have both latent and lytic programs (Strowig *et al.*, 2009; Chijioke *et al.*, 2013; Tsai *et al.*, 2013) and infected B cells are found mostly in the latency III program, as shown with immunohistochemistry staining for EBNA1, EBNA2 and LMP1, or with in situ hybridization staining for EBV-encoded RNAs (EBERs) (Cocco *et al.*, 2008; Strowig *et al.*, 2009). Depending on the dose administered, an asymptomatic persistent infection course, or a more acute IM-like infection can be seen (Yajima *et al.*, 2008). Even though lytic EBV-infected B cells are found in humanized mice, they are outnumbered by latently infected cells. Persistent infection does not depend on lytic replication, as an EBV mutant deficient for BZLF1 was still able to establish latent infection in humanized mice (Ma *et al.*, 2011; Chijioke *et al.*, 2013; Antsiferova *et al.*, 2014).

While humoral immune responses are weak in humanized mice due to inefficient germinal center organization and class switching, $CD4^+$ and $CD8^+$ T cells are reconstituted and both are important for the control of EBV (Strowig *et al.*, 2009; Yajima *et al.*, 2009). During EBV infection of HLA-A2 transgenic animals, T cells against both lytic and latent antigens are seen, even so often with dissimilar peptide specificities than in humans (Strowig *et al.*, 2009; Shultz *et al.*, 2010). EBV-specific $CD8^+$ T cells can produce IFNγ in response to cognate epitopes (Strowig *et al.*, 2009) and autologous LCLs (Traggiai *et al.*, 2004; Melkus *et al.*, 2006; Yajima *et al.*, 2008). Moreover, both $CD4^+$ and $CD8^+$ T cells isolated from EBV-infected animals are able to lyse LCLs (Strowig *et al.*, 2009). Thus, cell-mediated, but not antibody mediated immune responses to EBV can be modelled in humanized mice and their modification by MS predisposing genetic risk factors was analyzed in this thesis.

1.4 Aims of the study

In this PhD thesis, the broad aim was to investigate MS-associated risk factors in the context of EBV infection. In the **first** project, we investigated effects on EBV immune control in the context of the main genetic risk factor for MS, the HLA-DR15 MHC class II allele. In the **second** project, we aimed to generate an NSG mouse model transgenic for HLA-DR15, with which we could study in more detail results obtained in the first project, as well use this mouse model to investigate autoimmunity in the context of this MHC molecule. Finally, in the **third** project, we wanted to further look into several other genetic risk variants that have been implicated in the risk of MS. Using our model of EBV infection, we wanted to determine causal variants from a pre-selected list of SNPs, which could contribute to defects in EBV immune control associated with MS.

2 Attenuated immune control of the Epstein Barr virus in the context of the main genetic risk factor for multiple sclerosis

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H.Z., A.M., C.E., A.R., Y.D., C.G., J.R., D.M., N.C. and B.C. performed experiments. H.Z. and B.C. pooled and stratified data. H.Z., A.M., Y.D., D.M. and N.C. provided serum samples for IgM analysis. J.R. provided T cell cloning protocol and H.Z. performed T cell cloning and T cell clone experiments. G.F. and G.P. performed HLA typing. A.Z. and R.C. determined EBV titers. R.M. provided BLS cell lines. R.M. and J.L. provided valuable feedback. C.M. and B.C. supervised and C.M., B.C. and H.Z. designed the study. C.M. acquired funding.

2.1 Disclaimer

This thesis chapter is based on the following manuscript:

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Attenuated immune control of the Epstein Barr virus in the context of the main genetic risk factor for multiple sclerosis

Submitted manuscript.

2.2 Abstract

Immune responses to Epstein Barr virus (EBV) infection synergize with the main genetic risk factor HLA-DRB1*15:01 (HLA-DR15) to increase the likelihood to develop the autoimmune disease multiple sclerosis at least seven-fold. In order to gain insights into this synergy, we investigated HLA-DR15 positive human immune compartments after reconstitution in immune compromised mice (humanized mice) with and without EBV infection. We detected elevated activation of both $CD4^+$ and $CD8^+$ T cells in HLA-DR15 positive humanized mice at steady state, even when compared to immune compartments carrying the HLA-DRB1*04:01 (HLA-DR4) gene, which is associated with other autoimmune diseases. Increased $CD8^+$ T cell expansion and activation was also observed in HLA-DR15 positive humanized mice after EBV infection. Despite this higher immune activation, EBV viral loads were less well controlled in the context of HLA-DR15. Indeed, when we cloned HLA-DR15 or HLA-DR4-restricted $CD4^+$ T cells specific for EBV-transformed B cell lines (LCLs), HLA-DR15-restricted $CD4^+$ T cell clones recognized LCLs less efficiently and demonstrated cross-reactivity towards allogeneic target cells. These findings suggest that EBV as one of the main environmental risk factors and HLA-DR15 as the main genetic risk factor for multiple sclerosis synergize by priming hyperreactive T cell compartments, which then control EBV infection less efficiently and contain cross-reactive $CD4^+$ T cell clones.

2.3 Introduction

Multiple sclerosis is an inflammatory, demyelinating disease of the central nervous system (CNS). It affects around 2.5 million people worldwide and leads to substantial decline of sensory, motor, autonomic and neurocognitive functions. While considered to be an autoimmune, $CD4^+$ T cell-mediated disorder, its etiology still remains poorly understood (Sospedra and Martin, 2005).

Over the last few years, genome-wide association studies have provided great insights into the complex genetics behind multiple sclerosis, identifying over 100 genetic loci that contribute to the risk of acquiring the disease. The strongest association has been observed with the human leukocyte antigen (HLA) locus, and in particular, the HLA-DRB1*15:01 (HLA-DR15) allele in the *HLA-DRB1* gene. Other identified loci represent mainly immunologically relevant genes and particularly suggest a role for T helper cell differentiation and function in the disease pathogenesis (Sawcer *et al.*, 2011; Beecham *et al.*, 2013).

However, because the genetic background confers only partial risk for the disease, the role for environmental risk factors and their synergies with multiple sclerosis genetics have become increasingly apparent (Olsson *et al.*, 2017), especially in the first two decades of life (Gale and Martyn, 1995). Amongst several environmental factors implicated in multiple sclerosis, altered immune response to the Epstein-Barr virus (EBV), including immunopathology during the acute primary EBV infection known as infectious mononucleosis (IM), is associated with the disease and synergizes with HLA-DR15 resulting in a seven-fold increased risk for multiple sclerosis (Nielsen *et al.*, 2009). The mechanisms behind this synergy remain unclear.

EBV is a ubiquitous γ-herpesvirus infecting around 95% of the world adult population (Niederman, 1982). Following oral transmission (Niederman *et al.*, 1976), it starts a latent, growth transforming infection of B cells and persists in their memory compartment (Pattengale *et al.*, 1974; Babcock *et al.*, 1998). EBV is generally acquired early in childhood and leads to an asymptomatic primary infection at young age (Biggar *et al.*, 1978a). However, if acquired in adolescence or early adulthood, it can manifest as IM (Niederman, 1982), an acute illness presenting with fever, sore throat, lymphadenopathy and fatigue (Balfour *et al.*, 2013). The acute infection sparks a vigorous immune response, characterized by immensely expanded EBV-specific $CD8^+$ T cells and, to a lesser extent, NK cells (Odumade *et al.*, 2012; Balfour *et al.*, 2013; Chijioke *et al.*, 2013). Why IM is a phenomenon largely of late primary infection remains incompletely understood, however research points towards immunopathogenic and exaggerated T cell responses (Silins *et al.*, 2001; Abbott *et al.*, 2017).

Viral and infectious agents have been thought to play a role in multiple sclerosis for decades (Poskanzer *et al.*, 1963; Leibowitz *et al.*, 1966; Kurtzke, 1993), however, because EBV is distributed worldwide, the evidence supporting its role in the disease was puzzling at first. In 2006, Pohl and colleagues demonstrated that near-complete EBV seropositivity was seen in pediatric multiple sclerosis patients, compared to 72% of control children (Pohl *et al.*, 2006). Similar results were also shown in an earlier study (Alotaibi *et al.*, 2004) and in addition, adult multiple sclerosis patients all became EBV positive prior to disease onset, suggesting that multiple sclerosis risk is extremely low in EBV seronegative individuals, but increases sharply after EBV infection (Levin *et al.*, 2010). Indeed, higher anti-EBV antibody titers were observed before multiple sclerosis onset (Ascherio *et al.*, 2001) and are associated with its risk (Levin *et al.*, 2005). The genetic risk for elevated anti-EBV nuclear antigen 1 (EBNA1) IgG titers was also shown to positively correlate with the development of multiple sclerosis (Zhou *et al.*, 2016). In addition to the increase in risk following asymptomatic EBV infection, this risk is further increased after IM in adolescence or early adulthood (Thacker *et al.*, 2006; Handel *et al.*, 2010; Endriz *et al.*, 2017). IM and multiple sclerosis share not only similar patterns of geographical distribution, socioeconomic status and ethnicity, but also the period of adolescence as an important window for both diseases (Niederman, 1982; Gale and Martyn, 1995). Thus, rather than EBV infection itself, elevated T cell and antibody responses to EBV are associated with risk of multiple sclerosis.

Evidence suggests dysregulated adaptive immunity to be at the core of multiple sclerosis (Sospedra and Martin, 2005; Dendrou *et al.*, 2015). HLA class II molecules, such as HLA-DR15, are expressed on antigen presenting cells (Roche and Furuta, 2015). They present peptides derived from self or foreign proteins to $CD4^+$ T cells, which then support and strengthen cellular and humoral immune responses (Swain *et al.*, 2012). Indeed, in addition to the importance of $CD4^+$ T cells in multiple sclerosis pathogenesis, evidence from CNS-infiltrating immune cells in patients and B cell-depleting therapies suggests additional roles for $CD8^+$ T cells, B cells and autoantibodies in the disease (Babbe *et al.*, 2000; Hauser *et al.*, 2008; Hauser *et al.*, 2017), supported by or supporting autoimmune $CD4^+$ T cell responses.

Interestingly, EBV as a risk factor has also been shown to interact with HLA genes implicated in multiple sclerosis (Olsson *et al.*, 2017). Antibodies against specific domains of EBNA-1 in HLA-DRB1*15:01 positive individuals are associated with a markedly increased multiple sclerosis risk (Sundstrom *et al.*, 2009) and synergize with the susceptible HLA allele, as does IM (Sundqvist *et al.*, 2012). However, adequate models are missing to study these interactions. Using a humanized mouse model (huNSG) of EBV infection, we investigated the role of HLA-DR15 in the immune control of EBV. Using data accumulated in our laboratory over several years, we were able to stratify animals reconstituted with HLA-DR15 positive and

negative donors and investigate basal T cell activation before and after IM-like EBV infection, as well as T cell expansion, viral loads and responses of $CD4^+$ T cells cloned from these animals. With our *in vivo* and *in vitro* data, we have demonstrated that T cells from HLA-DR15 positive donors have higher basal activation levels. Despite enhanced T cell proliferation, EBV viral loads are elevated which suggests an impaired T cell-mediated immune control of EBV under the restriction of HLA-DR15. Importantly, we also find evidence for increased alloreactivity in $CD4^+$ T cell clones derived from HLA-DR15 individuals. Our data offers an insight into the gene-environment interactions that could lead or add to the perturbed T cell homeostasis in multiple sclerosis.

2.4 Results

2.4.1 HLA-DR15$^+$ donors reconstitute hyperactive T cells in huNSG animals

We first wanted to investigate the effect of HLA-DR15 positive donors on T cell activation at steady state in humanized NOD-scid IL2 receptor γ-chain-deficient (huNSG) mice, as the MHC class II molecule HLA-DR15 carries the strongest genetic risk for multiple sclerosis (Sawcer *et al.*, 2011). To this end, we combined and stratified data from more than 3500 mice spanning six years of reconstitution experiments (Fig. 4A and B). Briefly, following intrahepatic injection of human fetal liver-derived human hematopoietic progenitor cells (i.e. donor) shortly after birth, animals were routinely bled after 3 months to check for the reconstitution of human immune system components in peripheral blood at steady state. Animals reconstituted with HLA-DR15 positive donors demonstrated a higher activation of total T cells, $CD4^+$ and $CD8^+$ T cells when compared to animals reconstituted with HLA-DR15 negative donors (Fig. 4A). This T cell activation was mainly observed among $CD4^+$ T cells, which constitute around two thirds of the T cells after reconstitution. Furthermore, a higher activation frequency in the T cell compartment was also seen upon reconstitution with HLA-DR15 positive donors when compared with donors positive for HLA-DR4 (Fig. 4B), an MHC class II molecule implicated in other autoimmune diseases, including rheumatoid arthritis and type I diabetes (Fugger and Svejgaard, 2000; Noble and Valdes, 2011). These findings illustrate that HLA-DR15 hematopoietic progenitor cells reconstitute hyperactive T cells in the huNSG mouse model at steady state.

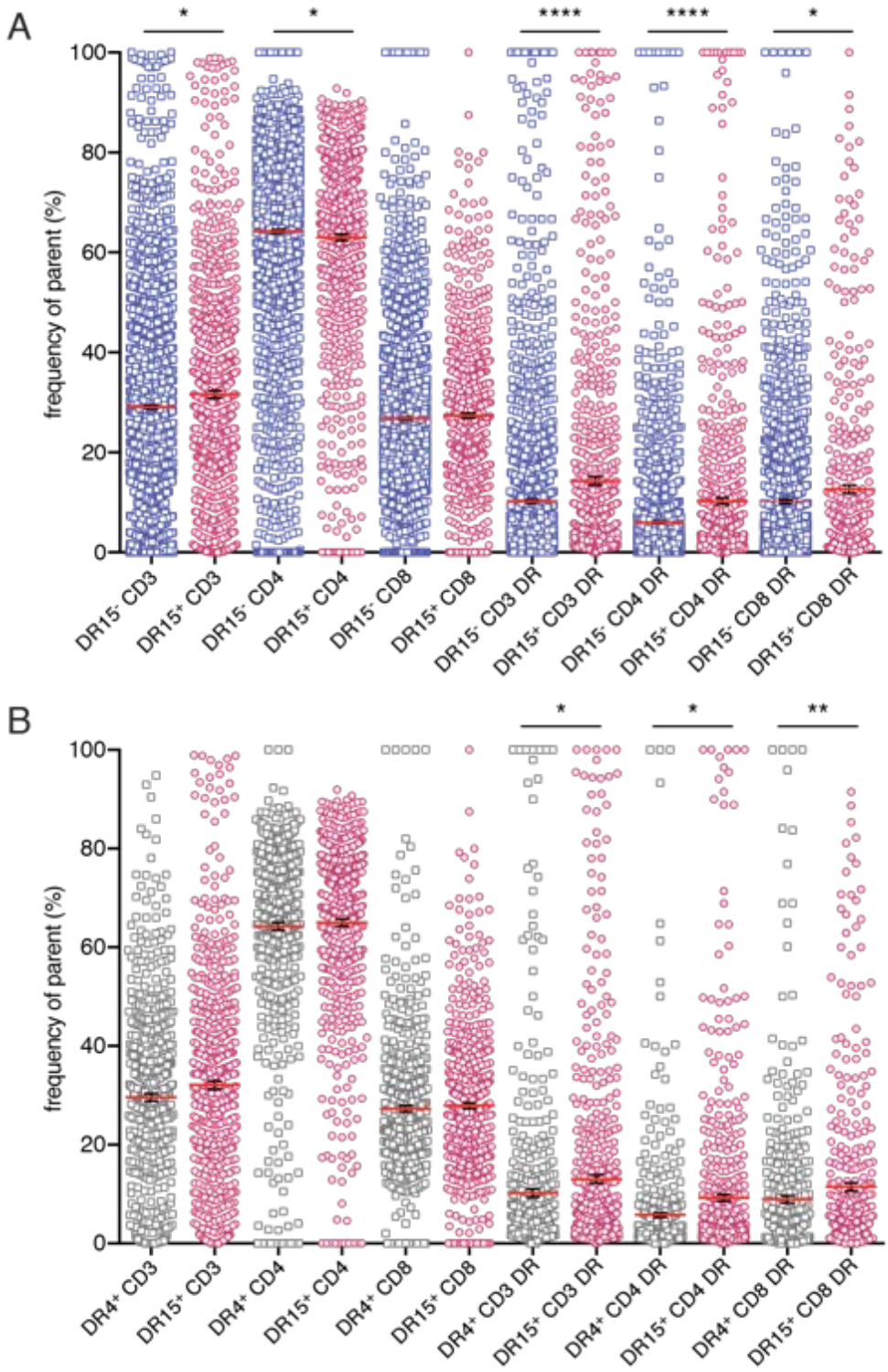


Figure 4: Higher T cell activation in HLA-DR15^{+} donor-reconstituted huNSG mice. Three months post reconstitution, animals were bled and analyzed for reconstitution of human immune system components in peripheral blood. Data was stratified into animals reconstituted with HLA-DR15 negative (DR15^{-}), HLA-DR15 positive (DR15^{+}) (**A**) and HLA-DR4 positive (DR4^{+}) (**B**) donors. T cell activation was analyzed by positive staining of HLA-DR (DR). Data shown are combined from > 96 (n of animals = 3579) (**A**) and 51 (n of animals = 1139) (**B**) donors. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, significant

comparisons are indicated and significance is based on the Mann-Whitney U test. Each symbol indicates one animal (mean ± SEM).

2.4.2 HLA-DR15 alters $CD8^+$ T cell frequencies and activation during EBV infection of huNSG mice

One of the hallmarks of IM is the vast expansion of $CD8^+$ T cells, which are key to the immune control of EBV (Callan *et al.*, 1996; Strowig *et al.*, 2009). As the huNSG mouse model has been successfully used to study IM-like EBV infection (Gujer *et al.*, 2015), we examined the expansion of T cells following EBV infection in animals reconstituted with donors positive or negative for HLA-DR15.

During these experiments, animals are infected with a high dose (10^5 infectious units) of the B95-8 strain of EBV and monitored for four to six weeks (Fig. 5A). We first wanted to investigate T cell expansion following EBV infection at time of sacrifice (Fig. 5B-I). Even though EBV infection induces a large expansion of $CD8^+$ T cells, we observed higher frequencies (Fig. 5B and 5C) and total numbers (Fig. 5D and 5E) in huNSG mice reconstituted with HLA-DR15 positive versus negative donors in both blood and spleen. While we didn't observe a difference in the frequencies of $CD4^+$ T cells (Fig. 5F and 5G) and total numbers in spleen (Fig. 5I), we did see higher total numbers of $CD4^+$ T cells in the blood of HLA-DR15-reconstituted animals (Fig. 5H). Higher total numbers of total T cells were also observed (Supplementary Fig. 1C and 1D).

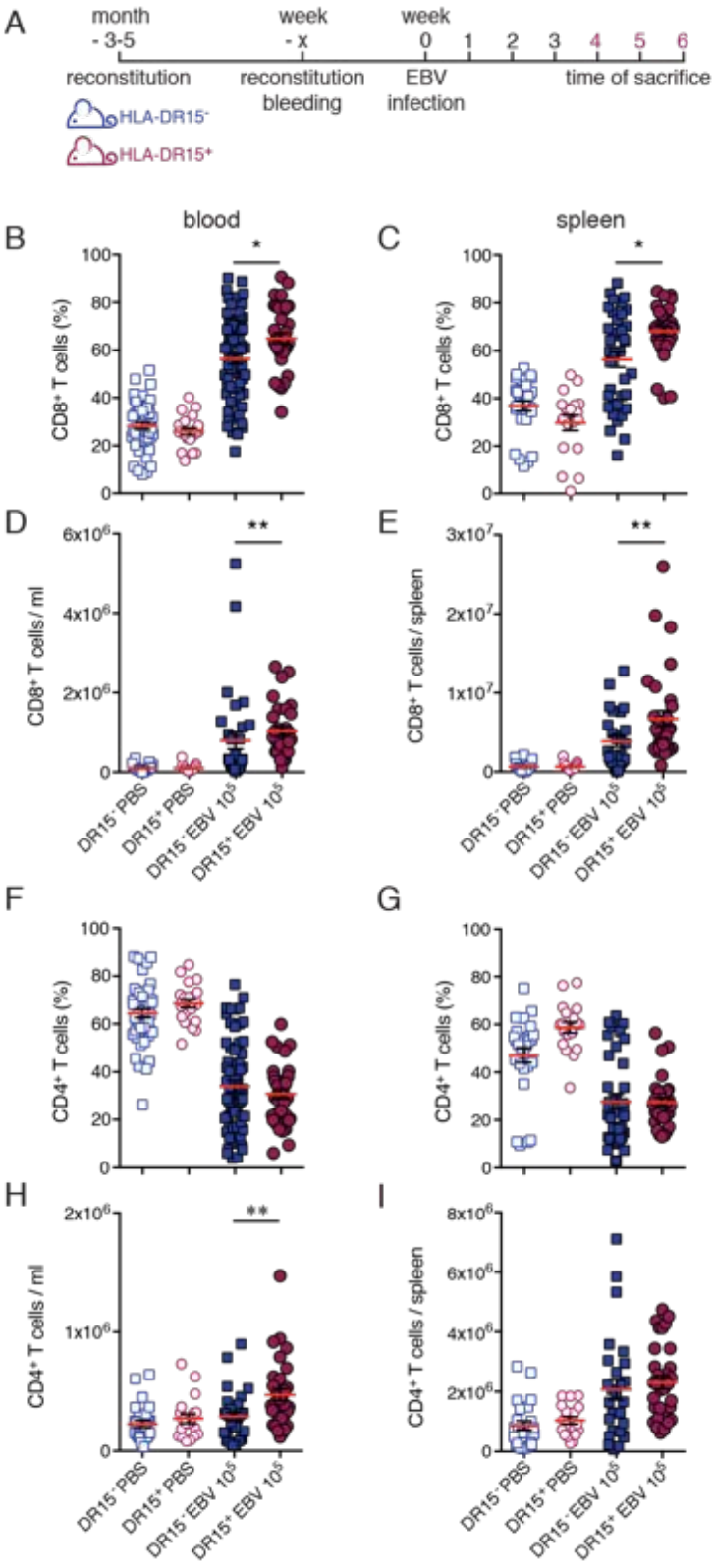


Figure 5: EBV-infected HLA-DR15$^+$-reconstituted mice have higher frequencies of CD8$^+$ T cells. Schematic depiction of analysed EBV infection experiments. After 3-5 months of human immune compartment reconstitution, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4-6 weeks (**A**). At time of sacrifice (4-6 weeks post infection), frequencies of CD8$^+$

T cells in blood and spleen (**B** and **C**), total numbers of $CD8^+$ T cells in blood and spleen (**D** and **E**), frequencies of $CD4^+$ T cells in blood and spleen (**F** and **G**), and total numbers of $CD4^+$ T cells in blood and spleen (**H** and **I**), respectively, were analysed and stratified into animals reconstituted with HLA-DR15 negative ($DR15^-$) and HLA-DR15 positive ($DR15^+$) donors. Data shown are combined from 11 to 24 experiments and contain 19-65 animals per group. * $p < 0.05$, ** $p < 0.01$, significant comparisons are indicated and significance is based on the Mann-Whitney U test. Each symbol indicates one animal (mean ± SEM).

In addition, there were higher numbers of activated total T cells (Supplementary Fig. 1E, 1F) and $CD8^+$ T cells (Fig. 6A and 6B) in blood and spleen of HLA-DR15-reconstituted animals. No differences were observed in the activation of the $CD4^+$ T cell compartment (Fig. 6C and 6D). These observations suggest a more robust, HLA-DR15-associated T cell proliferation and activation following EBV infection.

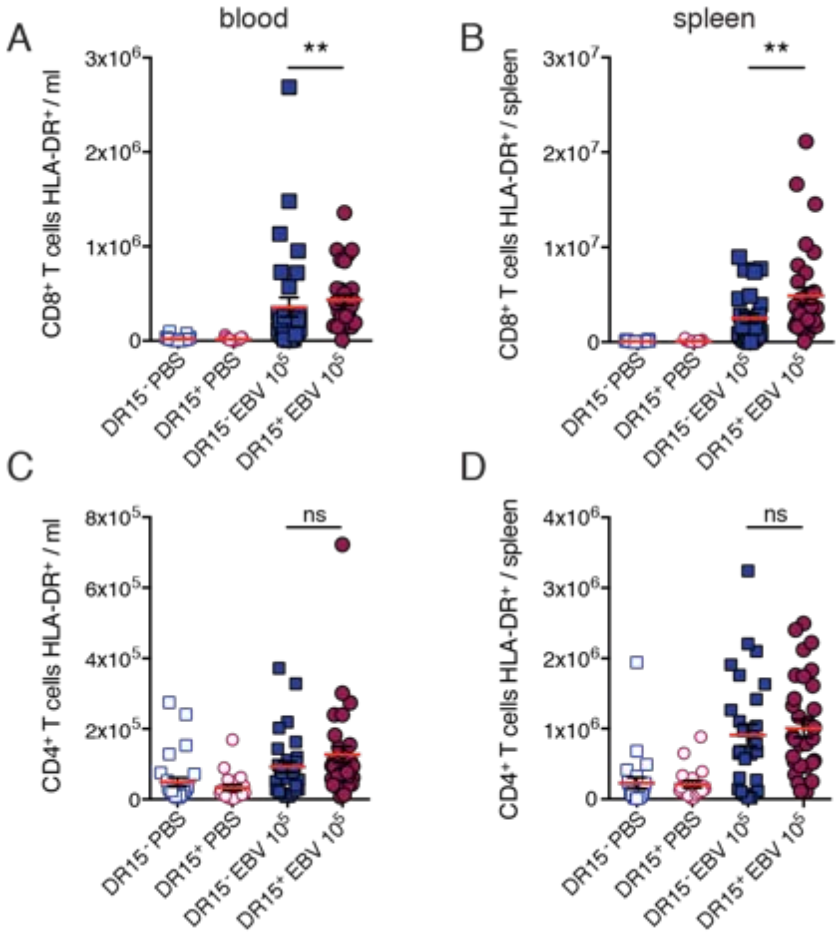


Figure 6: EBV-infected HLA-DR15$^+$-reconstituted mice have higher numbers of activated CD8$^+$ T cells. As indicated, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally. At time of sacrifice (4-6 weeks post infection), total numbers of activated CD8$^+$ T cells (**A** and **B**) and activated CD4$^+$ T cells (**C** and **D**) in blood and spleen, respectively, were analysed and stratified into animals reconstituted with HLA-DR15 negative (DR15$^-$) and HLA-DR15 positive (DR15$^+$) donors. T cell activation was analyzed by positive staining of HLA-DR. Data shown are combined from 11 to 13 experiments and contain 19-32 animals per group. * $p < 0.05$, ** $p < 0.01$, significant comparisons are indicated and significance is based on the Mann-Whitney U test. Each symbol indicates one animal (mean ± SEM).

2.4.3 Reduced EBV-specific immune control in HLA-DR15-reconstituted huNSG

In our huNSG model of EBV infection (10^5 infectious particles), blood viral DNA loads are usually detectable at three weeks post infection (Gujer *et al.*, 2015). Interestingly, we observed a higher EBV viral load at time of sacrifice in animals reconstituted with HLA-DR15 positive versus HLA-DR15 negative donors (Fig. 7A) or HLA-DR4 positive donors (Fig. 7B). It has previously been shown that CD8$^+$ T cell expansion and blood viral load are positively correlated with disease severity in IM patients (Balfour *et al.*, 2013). Indeed, we observed positive correlations between EBV viral loads in the blood and frequencies of activated CD8$^+$ T cells in the blood (Fig. 7C), as well as EBV viral loads in the spleen and frequencies of activated CD8$^+$ T cells in the spleen (Fig. 7D). Total numbers of activated CD8$^+$ T cells in blood and spleen also correlated with respective EBV viral loads (Supplementary Fig. 2A and 2B). This data, together with increased frequencies (Fig. 5B-E) and activation (Fig. 6A and 6B) of CD8$^+$ T cells, suggests a reduced EBV-specific immune control despite significant CD8$^+$ T cell expansion that is associated with HLA-DR15.

The humoral immune response has been challenging to study in the huNSG and other humanized mouse models. These animals lack normal lymph node development and have poor germinal center organization, thereby hampering B cell class switching and affinity maturation (Gujer *et al.*, 2015). Upon measuring serum anti-EBV nuclear antigen 1 (EBNA1) immunoglobulin M (IgM), we were, however, able to find a fraction of EBV-infected animals positive for these antibodies (Fig. 7E). The humoral anti-EBNA1 IgM titer from animals with a humoral response above threshold correlated with blood EBV viral loads (Fig. 7F), however due to the infrequency of these IgM responses, we were not able to compare HLA-DR15 positive and negative animals. Thus, EBV is less well immune controlled in the context of HLA-DR15; higher viral loads were observed despite elevated CD8$^+$ T cell expansion.

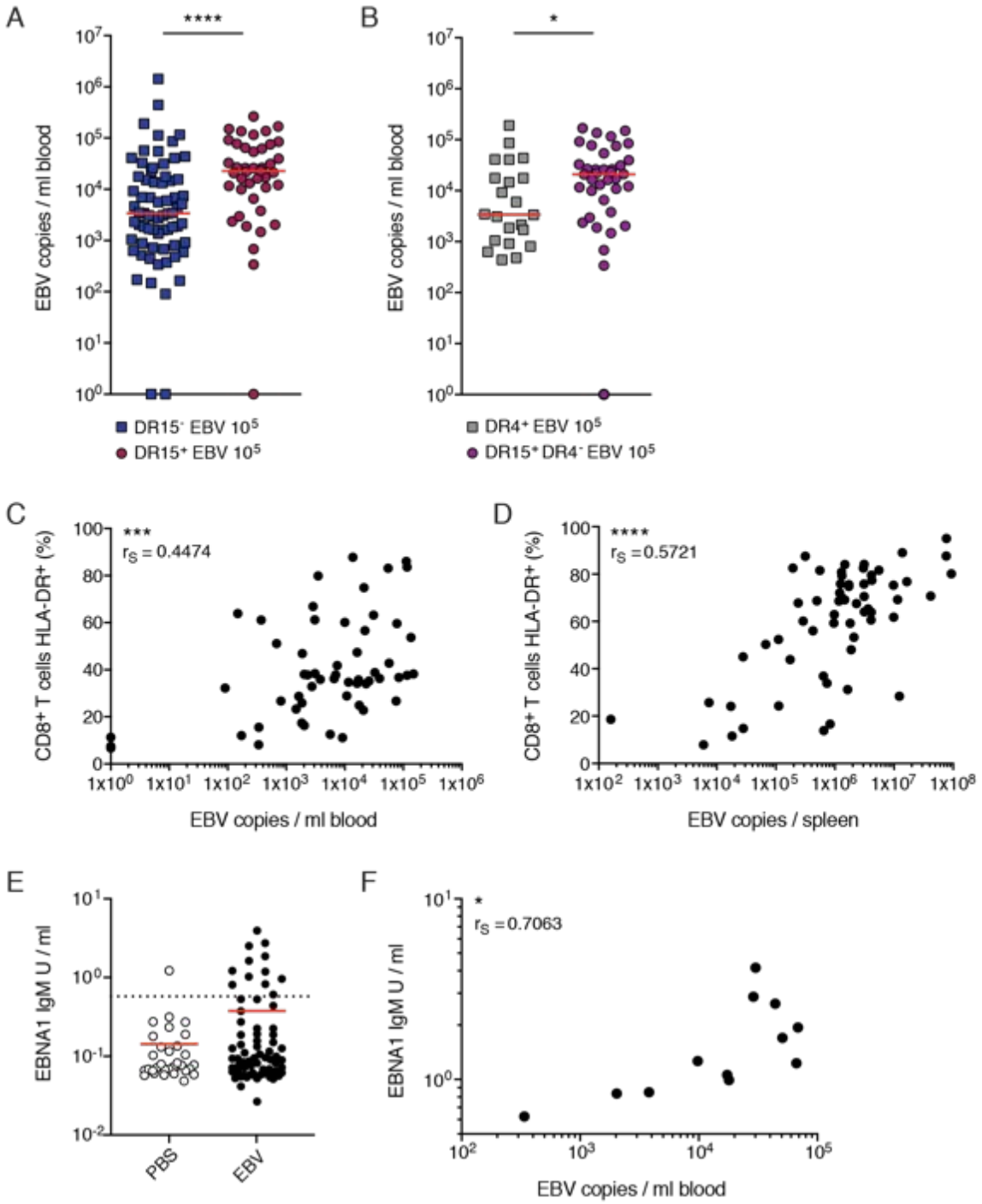


Figure 7: EBV viral loads in huNSG mice correlate with activated CD8$^+$ T cells and humoral IgM responses and are higher in HLA-DR15$^+$-reconstituted animals. Blood EBV viral loads at time of sacrifice (4-6 weeks post infection with 10^5 EBV infectious units) of animals reconstituted with HLA-DR15 positive (DR15$^+$), HLA-DR15 negative (DR15$^-$) (n = 42 and 70, respectively) (**A**), HLA-DR15 positive DR4 negative (DR15$^+$DR4$^-$) and HLA-DR4 positive (DR4$^+$) (n = 38 and 22, respectively) (**B**) donors. Red lines depict the median and significance is based on the Mann-Whitney U test (**A** and **B**). (**C**) Frequencies of activated CD8$^+$ T cells in blood were plotted relative to blood EBV viral loads (n = 56). (**D**) Frequencies of activated CD8$^+$ T cells in spleen were plotted relative to spleen EBV viral loads

(n = 60). T cell activation was analyzed by positive staining of HLA-DR (**C** and **D**). (**E**) Animals were injected with 10^5 EBV infectious units or PBS intraperitoneally. At time of sacrifice, serum was collected and analysed for anti-EBNA1 IgM antibodies via ELISA. Red lines depict the mean and dotted line represents threshold set at mean + 2x standard deviation of PBS animals (n PBS = 33, EBV = 71). (**F**) Levels of anti-EBNA1 IgM antibodies of animals above threshold were plotted relative to EBV viral loads in blood. Data shown are combined from 11 to 28 experiments. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, significant comparisons are indicated. All correlations were analyzed using the nonparametric Spearman correlation, which examines rank correlation. Each symbol indicates one animal.

2.4.4 Alloreactivity and lower specificity in HLA-DR15-restricted T cell clones

As T cells are essential for the immune control of EBV infection (Strowig *et al.*, 2009) and because our results suggest that EBV might be less well controlled under HLA-DR15 restriction, we examined T cell responses of individual $CD4^+$ T cell clones. For this purpose, $CD4^+$ T cell clones were cloned from EBV-infected, HLA-DR15 or HLA-DR4-reconstituted animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs; cells isolated from bare lymphocyte syndrome patients lacking MHC class II expression) transfected with single MHC class II molecules as targets. $CD4^+$ T cells that produced IFNγ in response to either HLA-$DR15^+$ or HLA-$DR4^+$ BLCLs were cloned (Fig. 8A). During the initial T cell clone screening for IFNγ production via ELISA, we observed BLCL-restricted IFNγ production in only 27% of HLA-DR15-restricted T cell clones, versus in 71% of HLA-DR4-restricted T cell clones tested (data not shown). Three HLA-DR15-restricted and two HLA-DR4-restricted $CD4^+$ T cell clones used in the assays demonstrated high levels of activation and expressed the transcription factor T-bet, suggestive of a T_H1 phenotype. All, except one HLA-DR15-restricted T cell clone, showed low expression of the transcription factor EOMES (Supplementary Fig. 3A-C).

To test their cytotoxic ability, the $CD4^+$ T cell clones were incubated together with their HLA matched or mismatched BLCLs as targets (HLA-DR15-expressing or HLA-DR4-expressing, depending on the genetic restriction of the T cell clone). While at 6 hours of incubation (Fig. 8B), the pattern is less evident, at 18 hours of incubation (Fig. 8C), HLA-DR4-restricted $CD4^+$ T cell clones killed their targets with higher specificity as HLA-DR15-restricted $CD4^+$ T cell clones which showed higher alloreactive responses. In addition, degranulation assessed by CD107a surface expression and the cytotoxic granule marker granzyme B were used to gain insight into the cytolytic potential of the T cell clones. Interestingly, HLA-DR4-restricted $CD4^+$ T cell clones degranulated very specifically, whilst HLA-DR15-restricted $CD4^+$ T cell clones not only degranulated less, but also demonstrated significant degranulation to the allogeneic target (Fig. 8D). HLA-DR15-restricted clones also displayed high granzyme

B expression when incubated with both HLA matched and mismatched targets, whilst HLA-DR4-restricted clones seemed to up-regulate granzyme B only upon recognition of the HLA matched target (Fig. 8E). In trying to isolate the specificity of the responses that we observed, the responses towards HLA mismatched BLCLs were subtracted from the recognition of HLA matched targets (Fig. 8B-E, lower panels) and further highlight above-mentioned results.

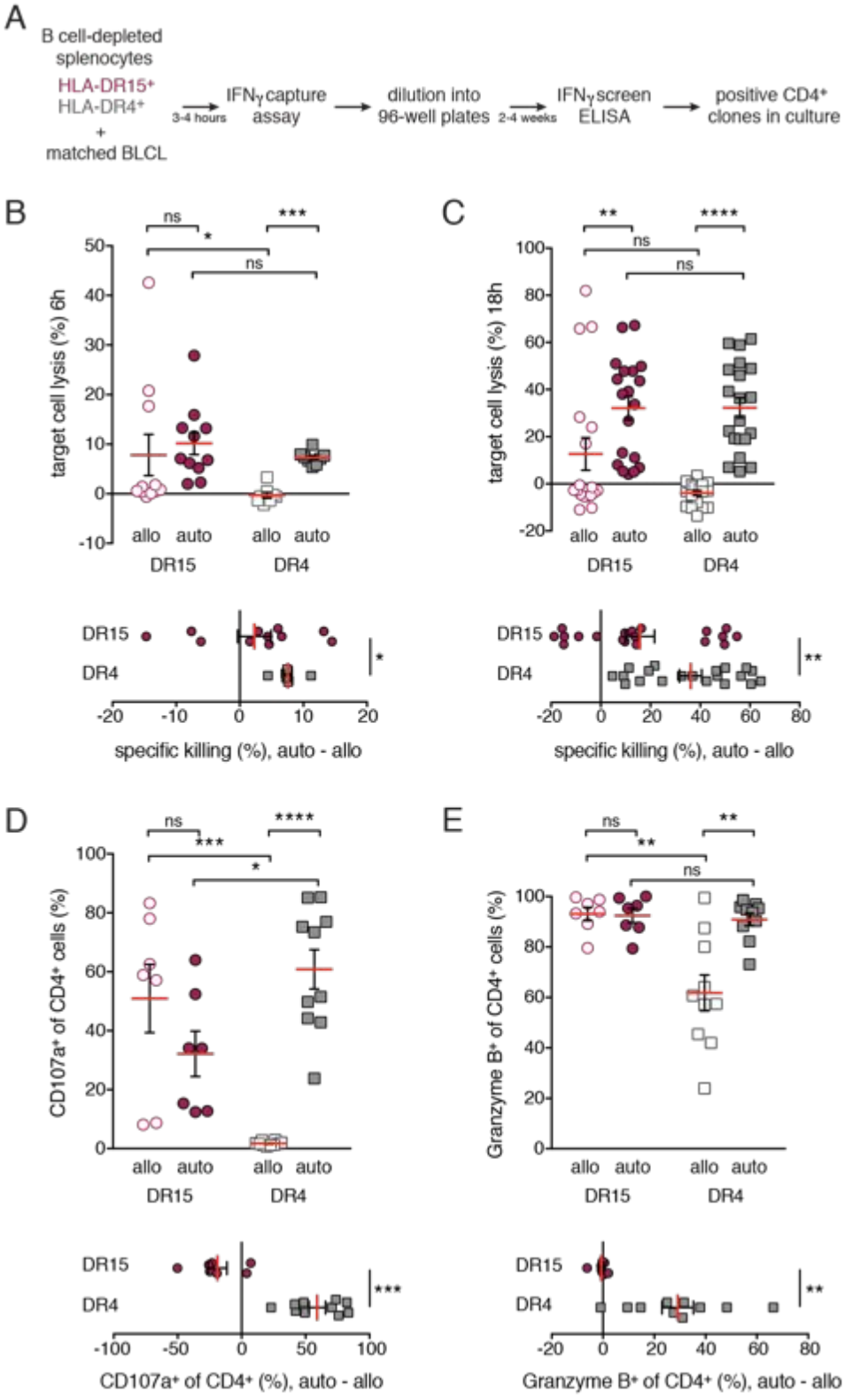


Figure 8: HLA-DR15-restricted EBV-infected B cell-specific CD4$^+$ T cell clones demonstrate higher alloreactivity in cytotoxicity. Schematic depiction of T cell cloning. CD4$^+$ T cell clones were cloned from EBV-infected animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs) transfected with single MHC class II molecules as targets using the IFNγ capture assay (**A**). HLA-DR15

(DR15) or HLA-DR4 (DR4)-restricted $CD4^+$ T cell clone responses are through target cell killing after 6 (**B**) and 18 hours (**C**), degranulation by marker CD107a surface upregulation after 8 hours (**D**) and granzyme B expression after 8 hours (**E**) in response to the allogeneic (allo) or autologous (auto) BLCL (HLA-DR15-expressing or HLA-DR4-expressing, depending on the genetic restriction of the T cell clone). To suggest the specificity of the response, allo values were additionally subtracted from auto values and included in bottom panels (**B**-**E**). Three HLA-DR15-restricted and two HLA-DR4-restricted $CD4^+$ T cell clones were used and data from 2 to 7 experiments are included. The mean ± SEM is depicted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, significant comparisons are indicated and significance is based on the Mann-Whitney U test.

In good agreement with these observations, intracellular cytokine staining for IFNγ (Fig. 9A), TNFα (Fig. 9B) and IL-2 (Fig. 9C) revealed stronger alloreactive responses from $CD4^+$ T cell clones restricted by HLA-DR15. Again, this is also evident when cytokine responses towards HLA mismatched BLCLs were subtracted from the recognition of HLA matched targets (Fig. 9A-C, lower panels). These data suggest weaker HLA-DR15-restricted $CD4^+$ T cell responses against EBV-transformed B cells, but also inflammatory responses that show tendencies towards alloreactivity. Thus, the HLA-DR15 genetic risk factor may restrict EBV specific $CD4^+$ T cell responses that recognize allogeneic target cells and that provide insufficient help to $CD8^+$ T cells in order to efficiently control EBV infection. This could lead to both IM-like lymphocytosis and promote cross-reactive autoimmune $CD4^+$ T cell stimulation.

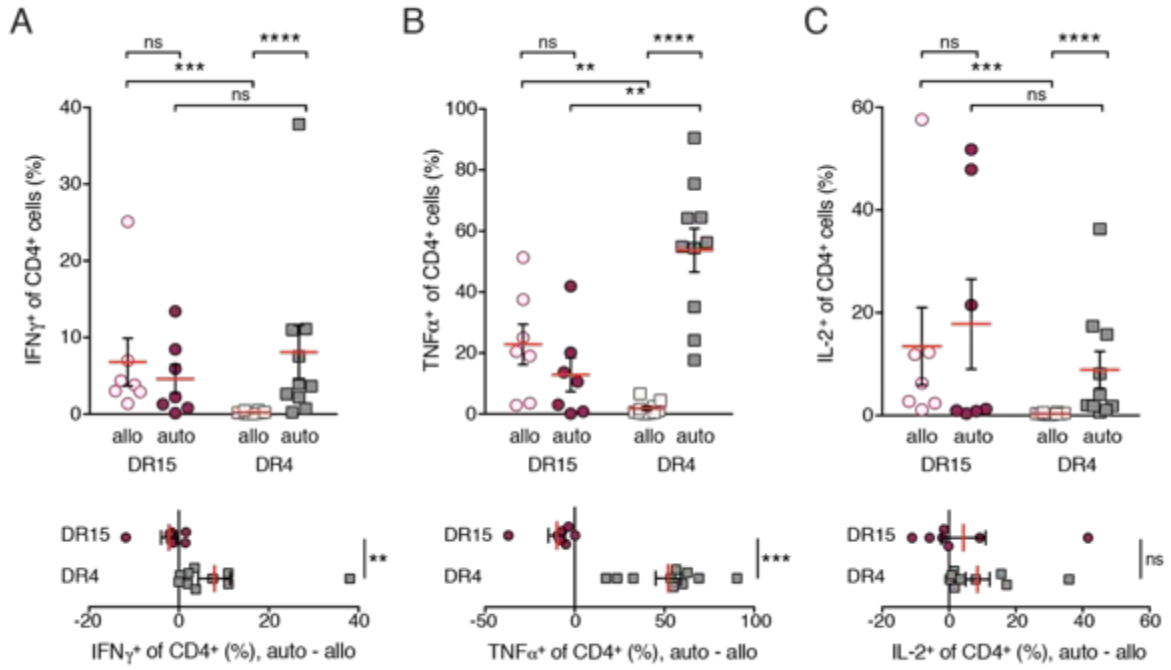


Figure 9: HLA-DR15-restricted EBV-infected B cell-specific CD4$^+$ T cell clones show higher unspecific cytokine production. CD4$^+$ T cell clones were cloned from EBV-infected animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs) transfected with single MHC class II molecules as targets. HLA-DR15 (DR15) or HLA-DR4 (DR4)-restricted CD4$^+$ T cell clone functionality was examined through the production of cytokines IFNγ (**A**), TNFα (**B**) and IL-2 (**C**) after 8 hours of incubation with allogeneic (allo) or autologous (auto) BLCL (HLA-DR15-expressing or HLA-DR4-expressing, depending on the genetic restriction of the T cell clone). Allo values were additionally subtracted from auto values and included in bottom panels (**A**-**C**). Three HLA-DR15-restricted and two HLA-DR4-restricted CD4$^+$ T cell clones were used and data from 7 experiments are included. Lines indicate mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, significant comparisons are indicated and significance is based on the Mann-Whitney U test.

2.5 Discussion

In vivo EBV infection of huNSG mice has allowed us to study the interaction between the main genetic risk factor for multiple sclerosis, HLA-DR15, with the main environmental risk factor, elevated EBV-induced immune responses (Olsson *et al.*, 2017). Similar to multiple sclerosis patients, we observed elevated EBV induced T cell responses in huNSG mice with HLA-DR15 positive immune compartments (Lunemann *et al.*, 2008a; Angelini *et al.*, 2013). These seemed to result from a higher constitutive T cell activation and increased $CD8^+$ T cell expansion due to less well controlled viral loads in connection with this particular MHC class II molecule. Furthermore, $CD4^+$ T cell clones from these EBV-infected huNSG mice that were selected for HLA-DR15-restricted LCL recognition displayed diminished immune recognition of EBV-infected B cells and higher cross-reactivity to HLA-DR mismatched targets. These findings point to both diminished immune control of EBV and its proinflammatory effects, as well as increased cross-reactivity, possibly resulting in molecular mimicry towards CNS autoantigens as possible pathogenic mechanisms. In this way, EBV-induced immune responses could synergize with HLA-DR15 to enhance multiple sclerosis.

Indeed, molecular mimicry between EBV and myelin-derived autoantigens has been proposed previously. Primarily, EBNA1 and lytic EBV antigen-specific antibody, in addition to $CD4^+$ T cell responses, are elevated in patients with multiple sclerosis and sometimes enriched in the CNS (Ascherio *et al.*, 2001; Lunemann *et al.*, 2008a; Lunemann *et al.*, 2008b; Levin *et al.*, 2010; Lunemann *et al.*, 2010; Angelini *et al.*, 2013; Dooley *et al.*, 2016; Erdur *et al.*, 2016). Some of these have indeed been suggested to cross-react with CNS autoantigens, like myelin basic protein, heterogeneous nuclear ribonucleoprotein L or septin-9 (Lunemann *et al.*, 2008b; Mameli *et al.*, 2014; Dooley *et al.*, 2016; Lindsey, 2017). This cross-reactivity could amplify T cell and antibody responses against select EBV gene products, including EBNA1, BFRF3 and BRRF2 (Lunemann *et al.*, 2008b; Dooley *et al.*, 2016; Lindsey, 2017), as well as lead to the local restimulation in the CNS to promote MS-associated lesion formation. Moreover, EBV might induce homing capacities in the respective cross-reactive lymphocyte populations to guide them to the CNS. In line with this, the chemokine CXCL10 was shown to be expressed by EBV-transformed cells, attracting EBV-specific T cells (Maggio *et al.*, 2002; White *et al.*, 2012) and was also found in the CSF of multiple sclerosis patients (Sorensen *et al.*, 1999).

Both B and T cell homing of cross-reactive specificities would however be a prerequisite of focusing the resulting autoimmunity on the CNS. Along these lines the $\alpha 4/\beta 1$ integrin has been found to be essential for CNS homing of T cells (Engelhardt and Ransohoff, 2012). In addition, the chemokine receptors CXCR3, CCR5, CCR6 and CCR7 have been

implicated in brain infiltration of T cells (Balashov *et al.*, 1999; Sorensen *et al.*, 1999; Arima *et al.*, 2012; Paroni *et al.*, 2017) and should be investigated for presence of cross-reactive lymphocyte populations. Cross-reactive T and B cells might then restimulate each other in the CNS of patients with multiple sclerosis to promote relapsing-remitting and progressive courses of the disease.

Such T cell stimulatory capacity of B cells in multiple sclerosis brains could be further augmented by EBV infection that increases co-stimulatory activity for T cell stimulation (Leung *et al.*, 2013). Along these lines, EBV-infected B cells have been detected in the meninges and lesions in the brains of multiple sclerosis patients (Serafini *et al.*, 2007; Moreno *et al.*, 2018), but these findings are heavily debated (Lassmann *et al.*, 2011). Considering EBV biology and life cycle, B cell stimulation of T cell responses in secondary lymphoid organs might be of great importance. As already mentioned, EBV establishes a life-long infection in memory B cells and by doing so, modulates their differentiation and function, and promotes their survival (Thorley-Lawson, 2001). Indeed, memory B cells have recently been shown to mediate the activation of autoreactive $CD4^+$ T cells (Jelcic *et al.*, 2018). It is plausible that EBV infects autoreactive B cells, or B cells that might present antigen to pathogenic, cross-reactive T cells over a long period of time. In addition, in genetically susceptible hosts, acute immune pathologies like IM could serve to decrease the threshold of T cell activation, facilitating pathogenic B-T cell interactions. The exaggerated EBV-specific T cell expansion during IM could expand T cells that have been selected in the thymus by self-peptides and will only undergo full activation under acute EBV infection to remain as memory T cells.

Indeed, B cell depleting therapies, which leave oligoclonal antibody production in the CNS and systemic antibody production by long-lived plasma cells in the bone marrow usually unaffected, have demonstrated beneficial effects in multiple sclerosis patients (Hauser *et al.*, 2017; Montalban *et al.*, 2017). Furthermore, adoptive transfer of EBV-specific *in vitro*-expanded T cell lines into patients with multiple sclerosis, even at advanced disease, has demonstrated some encouraging results (Pender *et al.*, 2014; Pender *et al.*, 2018). These T cells might eliminate an EBV-infected B cell compartment that stimulates autoreactive T cell responses, possibly even in the brain. Thus, inefficient immune control of EBV-infected B cells in the context of HLA-DR15 might allow for both priming of cross-reactive T cell responses and their local restimulation by EBV-infected B cells to propagate autoimmunity in the CNS of patients with multiple sclerosis.

2.6 Funding

This research was supported by supported by Cancer Research Switzerland (KFS-4091-02-2017), KFSP-Precision^{MS} of the University of Zurich, the Vontobel Foundation, the Baugarten Foundation, the Sobek Foundation, the Swiss Vaccine Research Institute, the Swiss MS Society, Roche and the Swiss National Science Foundation (310030B_182827 and CRSII5_180323).

2.7 Competing interests

The authors declare no competing financial interests.

2.8 Materials and methods

2.8.1 Animal work

NOD-scid $\gamma_c^{-/-}$ (NSG) animals with or without an HLA-A2 heavy chain transgene (Jackson Laboratory, Bar Harbor, Maine, USA) were maintained under specific pathogen-free conditions at the Institute of Experimental Immunology, University of Zürich. Newborn pups (1-5 days old) were sublethally irradiated with 1Gy and 5 to 7 hours later, intrahepatically injected with around 1.5-3 x 10^5 human fetal liver (Advanced Bioscience Resources)-derived and HLA typed $CD34^+$ hematopoietic progenitor cells. $CD34^+$ cells were isolated by magnetic bead separation based on manufacturer's instructions (Miltenyi Biotech) and frozen in liquid nitrogen. After twelve weeks, peripheral blood was collected via tail vein and analysed for human $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$ and HLA-DR^+ cells by flow cytometry. Animals 3 to 5 months old and engrafted with human immune cells were intraperitoneally injected with 10^5 EBV Raji infectious units (RIU) or PBS and monitored for up to 6 weeks. Animals used in each experiment were reconstituted from a single donor.

2.8.2 EBV

EBV strain B95-8 was produced in human embryonic kidney HEK293 cells (ATCC) containing a GFP-encoding wild type EBV BACmid (p2089; kind gift from H. Delecluse) (Delecluse *et al.*, 1998). Virus concentrates were titrated on Raji cells (ATCC) and GFP-expressing cells were analysed two days later by flow cytometry (FACSCanto II or LSR Fortessa, BD Biosciences) to determine the EBV RIU.

2.8.3 Enzyme-linked Immunosorbent assay (ELISA)

IFNγ in T cell clone supernatants was measured by the Human IFNγ ELISA development kit (Mabtech AB) according to the manufacturer's instructions. EBNA-1 IgM in huNSG mouse serum was measured by the Epstein-Barr virus EBNA-1 IgM ELISA (IBL International) following the manufacturer's instructions. The threshold to determine positive IgM values was set by the mean + 2x standard deviation of PBS animals.

2.8.4 T cell cloning

$CD4^+$ T cells were isolated from the splenocytes of EBV-infected animals (magnetic bead separation, Miltenyi Biotec) and stimulated with HLA-matched bare lymphocyte syndrome lymphoblastoid cell line (BLCLs; HLA-DR15 or HLA-DR4-transfected) for 3-4 hours at 37°C. IFNγ-producing cells were then selected for with the IFNγ Secretion Assay – Detection kit (PE), human (Miltenyi Biotech) and diluted into 96-well plates pre-filled with irradiated PBMC feeder cells and HLA matched BLCLs (20Gy and 60Gy, respectively). IL-2 (Peprotech) was added two days later and subsequently twice a week at 100-125 U/ml. Two to four weeks later, growing clones were screened with the Human IFNγ ELISA development kit (Mabtech AB) and IFNγ-producing clones were placed into culture. $CD4^+$ T cell clones were fed every two to three weeks with irradiated PBMC feeder cells and HLA matched BLCLs, together with 100-125 U/ml of IL-2.

For T cell clone killing assays, BLCLs were labelled with PKH26 (Sigma-Aldrich). $CD4^+$ T cell clones were then incubated alone, or with labelled matched or mismatched BLCLs at 5:1 ratio for 6 or 18 hours. TO-PRO-3 dye (Life Technologies) was used as a dead cell indicator. For degranulation experiments, $CD4^+$ T cell clones were incubated alone, or with matched or mismatched BLCLs at 1:1 ratio and labelled with CD107a (BD Biosciences). Cells were incubated for 2 hours before the addition of Brefeldin A (Sigma-Aldrich) and incubated for further 6 hours. For transcription factor expression experiments, $CD4^+$ T cell clones were incubated alone, or with matched or mismatched BLCLs at 1:1 ratio for 8 hours.

2.8.5 Flow cytometry

Whole blood was lysed with ACK lysing buffer (Gibco) and washed to obtain peripheral blood mononuclear cells (PBMCs). Spleens were physically dissociated through a 70 μM strainer and layered on Ficoll-Paque (GE Healthcare) for the separation of mononuclear cells by density gradient centrifugation. Total numbers of leukocytes were calculated from the white blood cell count measured by Beckman Coulter AcT diff Analyzer. PBMCs, splenocytes or $CD4^+$ T cell clones were stained with extracellular antibodies for 20 minutes at 4°C, washed and resuspended either in PBS or fixed in 1% paraformaldehyde (PFA). For intracellular staining, extracellular-stained samples were processed with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to manufacturer's instructions. For intranuclear staining, extracellular-stained samples were processed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Thermo Fisher Scientific) following the manufacturer's instructions. Live/dead Near IR (Invitrogen) or Zombie NIR (Biolegend) was used for live cell separation.

Antibody clones used in this study: CD45 (HI30, Biolegend), CD3 (OKT3, UCHT1, Biolegend), CD4 (OKT4, RPA-T4, Biolegend), CD8 (SK1, Biolegend), CD19 (HIB19, Biolegend), HLA-DR (G46-6, BD Biosciences, L243, Biolegend), EOMES (WD1928, eBioscience), T-bet (4B10, eBioscience), IFNγ (4S.B3, eBioscience), IL-2 (MQ1-17H12, eBioscience), TNFα (Mab11, BD Biosciences), Granzyme B (GB11, BD Biosciences), CD107a (H4A3, BD Biosciences).

Samples were acquired on the FACSCanto II or LSR Fortessa (BD Biosciences) and analysed using the Flowjo Software (Becton, Dickinson & Company).

2.8.6 Viral load quantification

Splenic tissue was processed for DNA isolation using the DNeasy Blood and Tissue Kit (QIAGEN) and total DNA from whole blood was extracted using the NucliSENS EasyMAG System (bioMérieux), following the manufacturer's instructions. TaqMan real-time PCR (Applied Biosystems) was used to quantify EBV DNA, with modified primers for the BamH1 W fragment (5′-CTTCTCAGTCCAGCGCGTTT-3′ and 5′-CAGTGGTCCCCCTCCCTAGA-3′) and a fluorogenic probe (5′-FAM CGTAAGCCAGACAGCAGCCAATTGTCAG-TAMRA-3′). All samples were analysed in duplicates.

2.8.7 HLA typing

DNA was extracted using the QIAGEN GmbH reagent, according to the manufacturer's instructions. Commercial HLA kits (Fujirebio Diagnostics Inc.) were used to identify the HLA alleles using a PCR sequence-specific oligonucleotide reverse assay. Data analysis was performed using the LIPA Interpretation Software (Fujirebio Diagnostics Inc.).

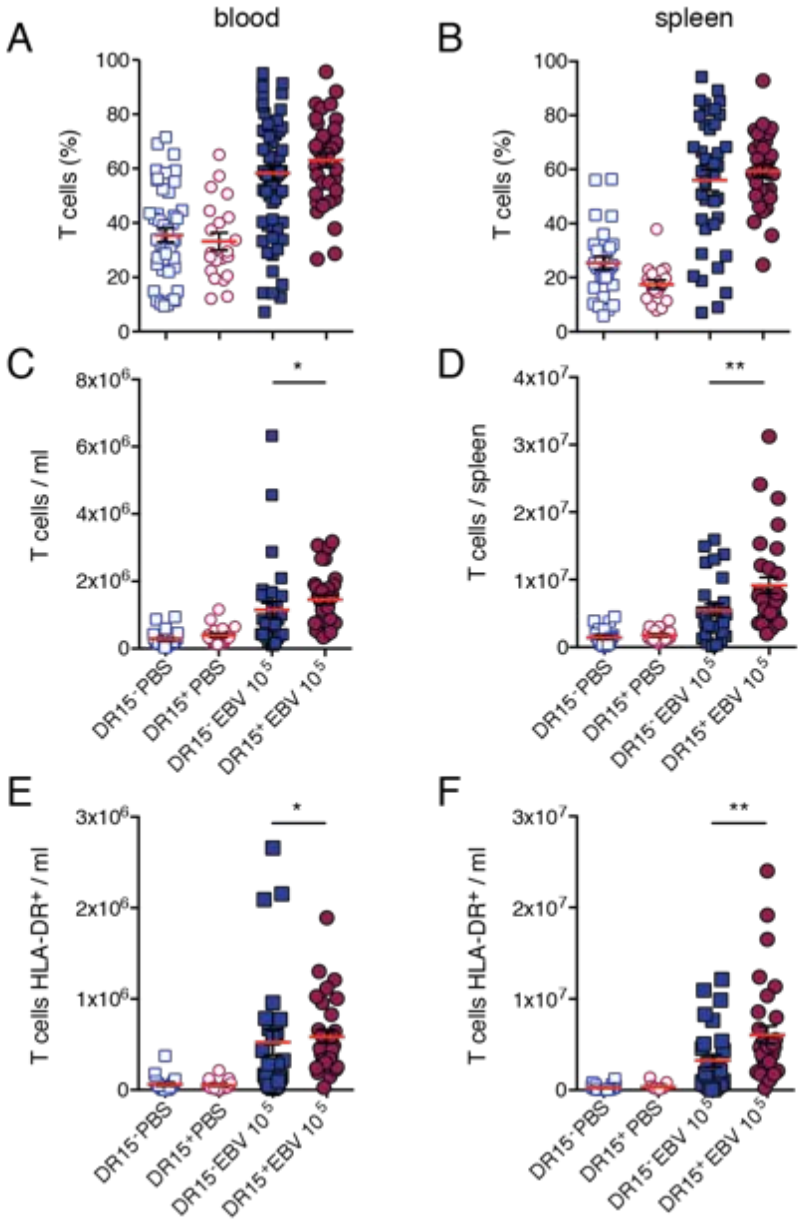
2.8.8 Statistical Analysis

Statistical analysis was performed with the GraphPad Prism Software. The Mann-Whitney U test was used to analyze unpaired data with a non-Gaussian distribution. Correlations on non-Gaussian distributed data were analyzed using the Spearman's rank correlation coefficient. The D'Agostino-Pearson omnibus normality test was used to determine normality of data. A p value < 0.05 was considered statistically significant.

2.8.9 Ethics statement

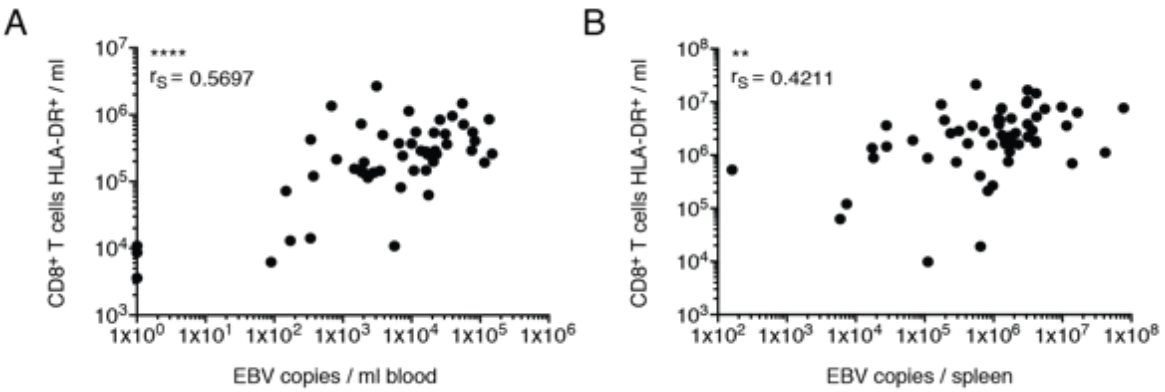
All animal experimentation was approved by the cantonal veterinary office of Zürich, Switzerland (protocols 148/2011, 209/2014 and 159/17) and conducted according to the Swiss Animal Welfare Act, Tierschutzgesetz (TSchG). All human sample studies were approved by the cantonal ethical committee of Zürich, Switzerland (protocols KEK-StV-Nr.19/08 and 2019-00837).

2.9 Supplementary data

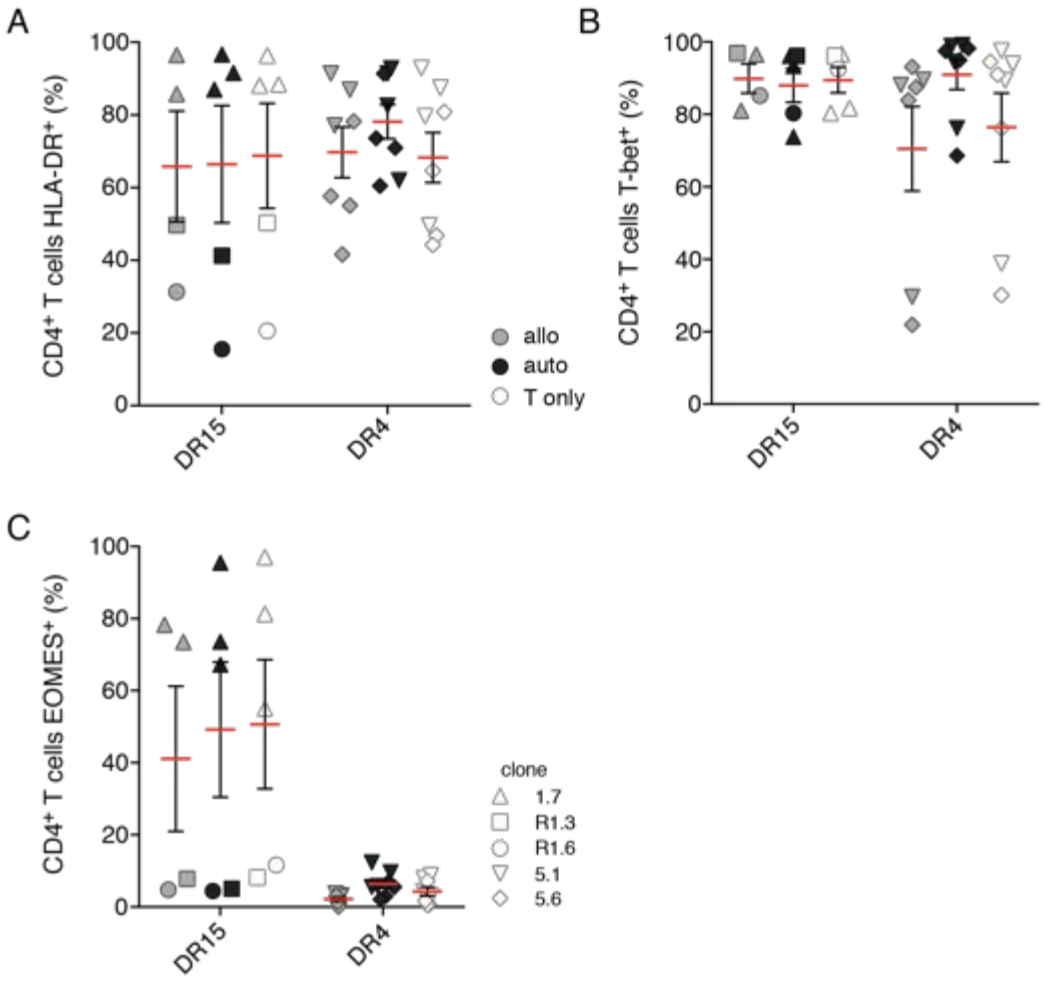


Supplementary Figure 1: Higher total numbers and total activated T cells in EBV-infected huNSG animals. As indicated in Fig. 2A, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4-6 weeks. At time of sacrifice (4-6 weeks post infection), frequencies of total T cells in blood and spleen (**A** and **B**), total numbers of T cells in blood and spleen (**C** and **D**) and total numbers of activated T cells in blood and spleen (**E** and **F**), respectively, were analysed and stratified into animals reconstituted with HLA-DR15 negative ($DR15^-$) and HLA-DR15 positive ($DR15^+$) donors. T cell activation was analyzed by positive staining of HLA-DR (**E** and **F**). Data shown are combined from 11 to 24 experiments and contain 19-65 animals per group. * $p < 0.05$, ** $p < 0.01$,

significant comparisons are indicated and significance is based on the Mann-Whitney U test. Each symbol indicates one animal (mean ± SEM).



Supplementary Figure 2: Activated CD8$^+$ T cells correlate to EBV viral load. (**A**) Total numbers of activated CD8$^+$ T cells in blood were plotted relative to blood EBV viral loads (n = 52). (**B**) Total numbers of activated CD8$^+$ T cells in spleen were plotted relative to spleen EBV viral loads (n = 56). T cell activation was analyzed by positive staining of HLA-DR (**A** and **B**). Data shown are combined from 11 experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, significant comparisons are indicated. All correlations are analyzed using the nonparametric Spearman correlation, which examines rank correlation. Each symbol indicates one animal.



Supplementary Figure 3: CD4$^+$ T cell clones are highly activated and are T-bet$^+$. CD4$^+$ T cell clones were cloned from EBV-infected animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs) transfected with single MHC Class II molecules as targets. CD4$^+$ T cell clones were incubated for 8 hours alone (T only), with matched (auto) or mismatched (allo) BLCL and their activation analyzed by positive staining for HLA-DR (**A**), T-bet (**B**) and EOMES (**C**) transcription factor expression are shown. Data shown are combined from 4 experiments and different symbols correspond to individual CD4$^+$ T cell clones.

3 HLA-DR15 transgenic NSG mouse

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H.Z. designed the construct, performed cloning and PCR experiments. J.vB. and D.K. performed pronuclear injection. H.Z. and F.L. bred colony and reconstituted animals. F.L. captured histology images. C.M. and B.C. supervised and C.M., H.Z. and B.C. designed the study. C.M. acquired funding.

3.1 Introduction

MS is an immune-mediated, demyelinating disease of the CNS and the main cause of neurological disability in young adults (Sospedra and Martin, 2005). The hallmark of the disease are inflammatory lesions within the brain and spinal cord, which are infiltrated by immune cells, mostly T cells (Dendrou *et al.*, 2015). The etiology of the disease is not yet completely understood. It clusters with other complex genetic diseases, characterized by moderate disease-risk heritability and intricate gene-environment interactions (Oksenberg *et al.*, 2008). There are hundreds of genetic and environmental risk factors that have been implicated in this disease.

MHC molecules present extracellular or intracellular peptides resulting from lysosomal or proteasomal degradation to T cells and are essential for the start of the adaptive immune response (Cresswell, 2019). As is the case with many autoimmune diseases, the HLA gene cluster on chromosome 6p21.3 coding for MHC, is the strongest genome-wide susceptibility locus for MS (Sawcer *et al.*, 2005). From this HLA cluster, the MHC class II haplotype, which consists of the alleles HLA-DRB1*15:01 (DR2b, or HLA-DR15), HLA-DRB5*01:01 (DR2a), HLA-DQA1*01:02 and HLA-DQB1*06:02 existing in linkage disequilibrium, bears the greatest effect on risk (Sawcer *et al.*, 2014). It has become apparent that the HLA-DR15 allele drives the association and increases the risk of MS 3-fold (Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Patsopoulos *et al.*, 2013). Interestingly, the HLA molecules have been shown to synergize with environmental risk factors, such as EBV, to further increase MS risk (Olsson *et al.*, 2017). The exact mechanisms behind HLA-DR15 association with MS and its influence on the interaction with EBV are important questions in MS.

Transgenic and humanized mice have been able to provide some evidence. In one of these models, animals transgenic for HLA-DR15, MBP-specific TCR and human CD4 co-receptor developed CNS inflammation and demyelination upon administration of MBP peptide plus adjuvant (Madsen *et al.*, 1999). In our previous study, we have used humanized NSG mice reconstituted with human $CD34^+$ hematopoietic progenitor cells (donors) isolated from human fetal livers to investigate T cell responses in animals reconstituted with HLA-$DR15^+$ donors at steady state and in EBV infection.

In this chapter, we aimed to create a HLA-DR15 transgenic mouse model on the NSG mouse background, in order to study in more detail HLA-DR15-restricted T and B cell responses in the context of cognate antigen presentation on mouse myeloid cells, as well as examine any resulting signs of autoimmunity. Additionally, this animal model could serve as a recipient and vessel to study MS patient-derived $CD4^+$ T cell clone responses and homing.

3.2 Results

3.2.1 Constructing and validating the HLA-DR15 gene and plasmid

As HLA-DR15 is the strongest genetic risk factor for multiple sclerosis (Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Patsopoulos *et al.*, 2013), and because we work with NSG mice, we were interested in making an HLA-DR15 transgenic mouse strain of the same background. We created the HLA-DR15 transgene by joining the sequences of the alpha chain DRA*01:01 via the P2A linker to the beta chain DRB1*15:01 (sequences in Appendix 2). We engineered the construct with EcoR1 flanking sites in a kanamycin resistant plasmid (pMK-T + HLA-DR15, Fig. 10A). We incorporated the previous work of Kouskoff and colleagues, where they describe a cloning vector pDOI-5 that is able to translate genetic material under the mouse MHC class II promoter (Eα promoter) (Kouskoff *et al.*, 1993). This ampicillin resistance vector contains the sequence of the Eα promoter, followed by the sequence of a rabbit β-globin gene, within which is an EcoR1 site (Fig. 10B).

To sequence the pMK-T + HLA-DR15 plasmid, we designed three overlapping forward primers spanning the length of the gene (Fig. 11A). The sequence on the plasmid matched the ordered sequence. Similarly, in order to sequence the pDOI-5 plasmid, we used the Eα promoter sequence to design four overlapping primers (Fig. 11B). With the sequence from primer 3 (EαP3_F), which ran into the rabbit β-globin gene, we were able to design the fourth primer in the β-globin gene (EαP4_F).

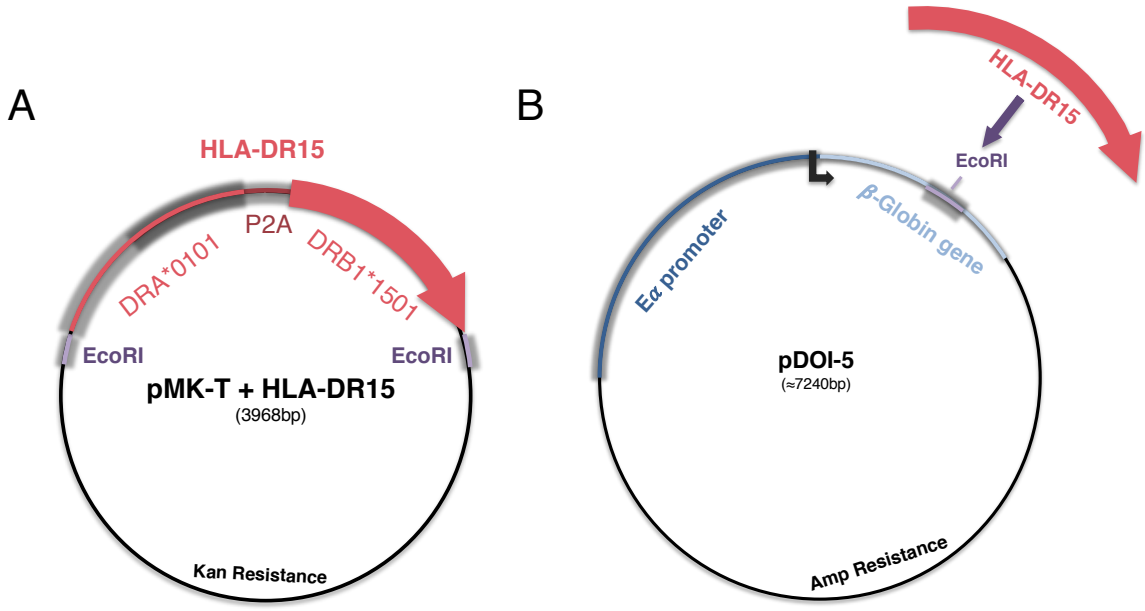


Figure 10. Schematic of plasmids used for HLA-DR15 transgenic mouse. (**A**) HLA-DRA α-chain was joined with P2A linker to HLA-DRB β-chain, flanked with EcoRI sites and inserted into a kanamycin resistance backbone. (**B**) The pDOI-5 plasmid has an ampicillin resistance backbone, contains an Eα promoter followed by a rabbit β-globin gene containing an EcoRI site. After EcoRI restriction, our HLA-DR15 gene fits into the rabbit β-globin gene.

Upon the restriction digest of our pMK-T + HLA-DR15 plasmid and the pDOI-5 plasmid with EcoRI, we were able to insert the cut-out HLA-DR15 gene into the pDOI-5 plasmid (Fig. 10B). In order to determine if the HLA-DR15 gene was inserted and in the correct orientation, we designed an additional fifth primer starting in the rabbit β-globin gene and spanning into the inserted HLA-DR15 (Fig. 11B). Like this, we sequenced 10 colonies, two of which had the insert in the correct orientation and proceeded with colony 10 for further studies.

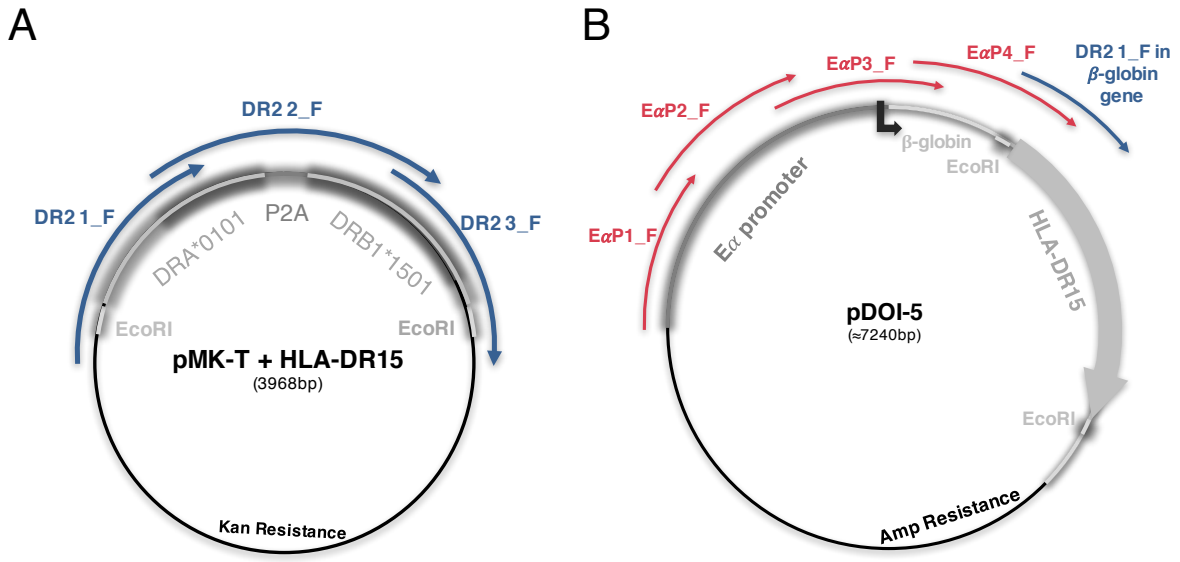


Figure 11. Schematic of plasmid primer design. (**A**) Three overlapping primers designed to sequence the length of the HLA-DR15 gene-containing plasmid. (**B**) Five overlapping primers designed to sequence the length of pDOI-5 plasmid, both with and without inserted HLA-DR15 gene.

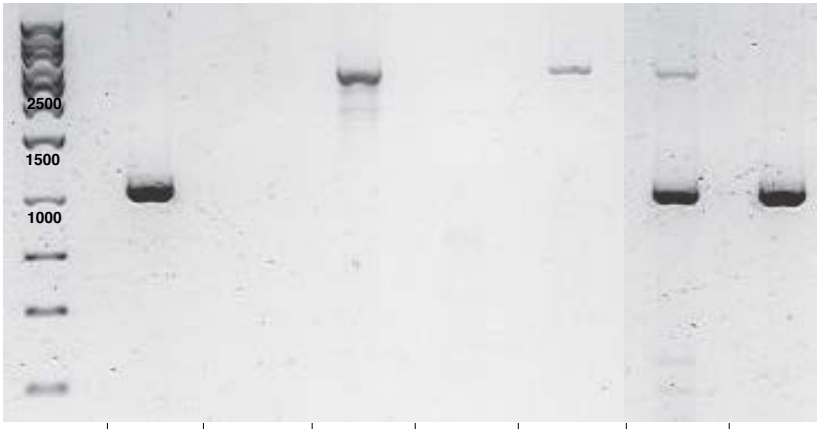
Next we developed a PCR method capable of distinguishing the plasmid in a sample. We first designed primers for a house-keeping gene; β-actin, to use as a control. We isolated DNA from an NSG mouse and/or spiked in the plasmid and added primers recognising the plasmid or primers recognising β-actin. While the β-actin primers did not appear to have

worked, we observed that the plasmid was recognised by primers designed to bind the plasmid, whether it was spiked into NSG DNA or was alone in the reaction (Fig. 12A). To verify the result with a second house-keeping gene (GAPDH), we observed that GAPDH primers detect the gene but not the plasmid. The plasmid is recognised by plasmid primers when it is spiked into NSG DNA, or alone. The plasmid primers also do not bind NSG DNA alone. When both plasmid primers and GAPDH primers are added to the plasmid-spiked DNA, both bands for the plasmid and GAPDH are seen (Fig. 12B). With this, we were able to detect the 100-fold diluted plasmid in a reaction with NSG DNA.

A

NSG DNA	+	+	+	+	
plasmid		+	+	+	+
plasmid primers	+	+		+	+
β actin primers	+	+	+		+

B



NSG DNA	+			+	+	+	+
plasmid *		+	+		+	+	+
plasmid primers			+	+	+	+	
GAPDH primers	+	+				+	+

*100 fold diluted

Figure 12. Specific binding of plasmid primers. Plasmid primer binding for genotyping was validated by the indicated combinations of plasmid, plasmid primers, DNA isolated from NSG mouse and primers towards house-keeping genes; β-actin (**A**) or GAPDH (**B**). Plasmid was isolated from bacterial colony 10 with gene insertion in correct orientation. A 1 kb ladder was used and band size of plasmid is 1100 (**A**) and 2700 (**B**) base pairs.

As the next step, we wanted to excise the Eα promoter with HLA-DR15 transgene out of the pDOI-5 plasmid. For this purpose, the pDOI-5 plasmid carries four BglI restriction sites and therefore this restriction enzyme can be used to liberate our sequence of interest from the plasmid (Fig. 13A). Of note, one of those BglI sites lies within the Eα promoter, however this was mentioned not to have an impact on gene expression (Kouskoff *et al.*, 1993). After a restriction digest with BglI and dephosphorylation with the Antarctic phosphatase enzyme, we ran the product on a gel and observed the expected four bands, with the top band (around 5500bp) representing our product of interest. Many BglI restrictions were performed to get the appropriate DNA amount of the linearized product. The DNA was sent to the laboratory of Dr. Johannes vom Berg and Dr. Thorsten Buch at the LTK, who super-ovulated NSG females and injected the linearized product, containing the Eα promoter followed by HLA-DR15 gene, into the oocytes of these animals.

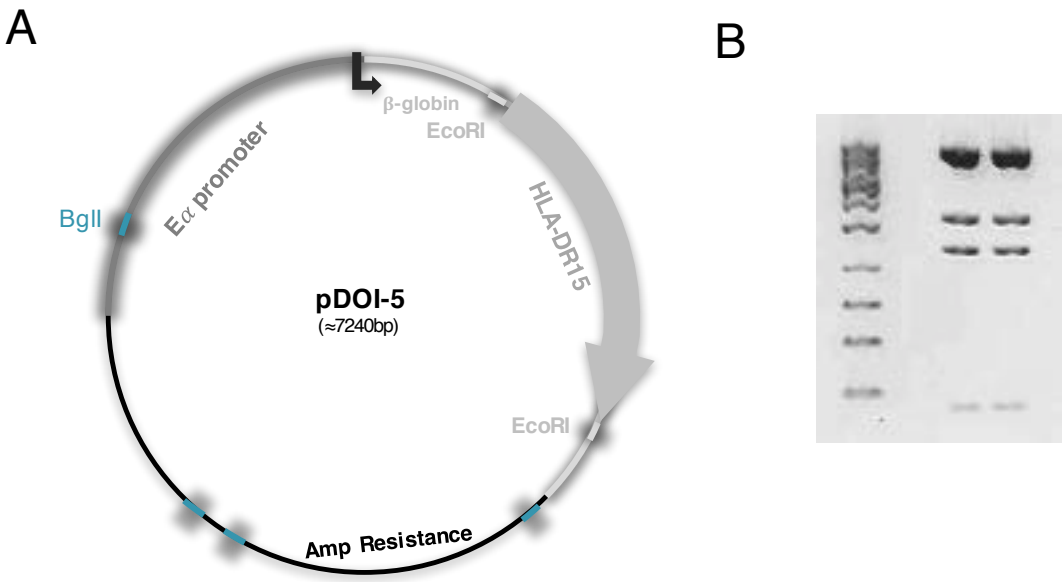


Figure 13. BglI digestion of p-DOI5. (**A**) Schematic of four BglI sites on the pDOI-5 plasmid. (**B**) Four bands after BglI restriction and dephosphorylation with Antarctic phosphatase. 1 kb ladder is used.

3.2.2 Gene expression and function

A genotyping protocol was optimized to genotype resulting founders from the pronuclear injections. While the DNA from positive animals and plasmid alone generate a band in the genotyping PCR, negative animals show no band (Fig. 14A). Even though the first round was unsuccessful, the second round yielded several HLA-DR15 positive pups, out of which two were selected for breeding and a colony was setup and housed in mouse facilities in Irchel and Schlieren. Upon staining for human MHC class II on spleen histology sections, we observed that while an NSG mouse does not show any expression (Fig. 14B), an HLA-DR15 transgenic NSG mouse shows high levels of expression across the tissue (Fig. 14C). Thymic and brain sections showed similar results (data not shown).

After verifying *ex vivo* expression of HLA-DR15 in our transgenic NSG mouse line, we were next interested in examining their reconstitution. We reconstituted similar numbers of NSG and NSG HLA-DR15 transgenic pups with human fetal liver-derived $CD34^+$ progenitor cells. After monitoring the animals for three months, we performed a bleeding and while the NSG animals showed average reconstitution (Fig. 14D), surprisingly and unfortunately, our HLA-DR15 transgenic NSG animals had 0% human $CD45^+$ cells in their peripheral blood. These data possibly suggest an incorrectly or incompletely inserted transgene DNA or insertion into an important gene that increases resistance to human immune system component reconstitution. Further characterization was done by Fabienne Läderach and will be presented in her master thesis.

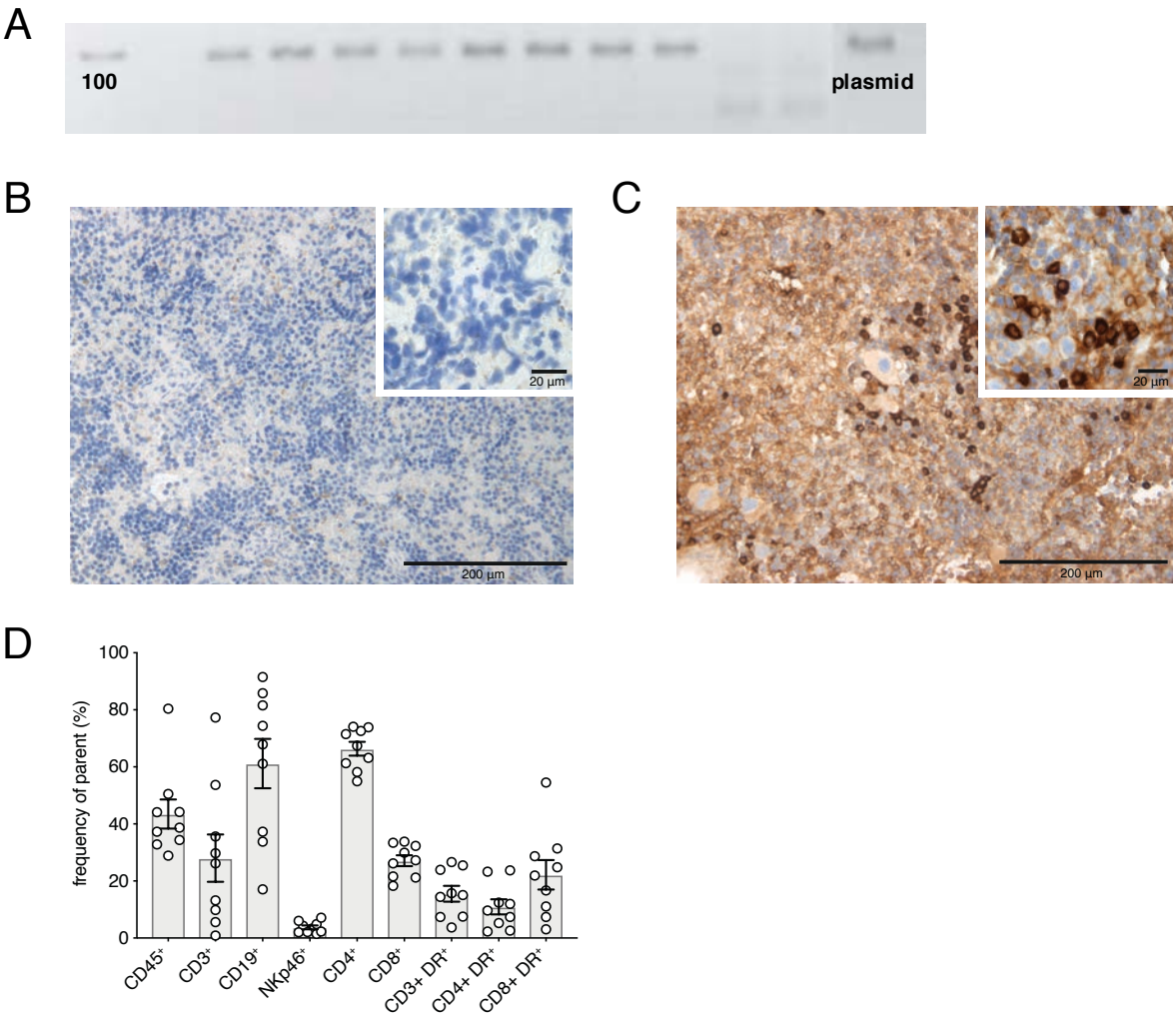


Figure 14. High expression of HLA-DR15 in transgenic NSG mice. (**A**) Genotyping of HLA-DR15-transgenic NSG mice. Eight animals shown are positive for transgene, two as negative. Plasmid isolated from bacterial colony 10 with gene insertion in correct orientation is used as control. 100 bp band of ladder is displayed. Histological spleen sections of NSG (**B**) and HLA-DR15-transgenic NSG (**C**) mouse. Sections were stained with human MHC class II and images were obtained at 20x and 100x magnification. Scale bars 200 µm, insert 20 µm. (**D**) Composition of human immune cells in peripheral blood of NSG animals three months after reconstitution with $CD34^+$ hematopoietic progenitor cells. One point indicates one animal, bar graph indicates the mean and error bars indicate the SEM.

3.3 Discussion

HLA-DR15 is the strongest genetic risk factor for MS (Sawcer *et al.*, 2011; Beecham *et al.*, 2013). Studies on patient samples and through GWAS studies have been able to shed light on the roles of this MHC class II molecule on disease pathogenesis. Nevertheless, having a reliable animal model to study the roles of HLA-DR15 in human T cell selection, antigen presentation, APC - T cell interaction and HLA-DR15-restricted T cell responses would be valuable.

In this study, we generated a transgenic mouse model of the NSG background carrying the HLA-DR15 allele of the MHC class II molecule with the aim of studying differences in reconstitution, T cell activation and specificity and T and B cell functionality. Furthermore, we wished to examine differences in EBV immune control and any signs of autoimmunity in these animals. The transgene was designed to contain the HLA-DRA*01:01 α-chain and the HLA-DRB1*15:01 β-chain and to insert into the genome at random. After the transgene injection into the oocytes of NSG females, we received animals positive for the transgene, which were set up to breed. Even though we observed the expression of human HLA-DR in these animals, unfortunately they did not reconstitute with human immune system components like control NSG mice.

Introducing MHC class I or class II molecules into mice of different strains has been done in several studies. In efforts to gain insights into MS and CNS pathology, the DBAxC57BL/6 mouse model was used to express HLA-DR15, together with an MBP-specific T-cell receptor and the human CD4 co-receptor. In this model, transgenic animals developed CNS inflammation and demyelination under the administration of the MBP peptide with adjuvant and pertussis toxin, as well as spontaneous disease in a small fraction of animals (Madsen *et al.*, 1999). In the same model transgenic only for HLA-DR15, the mouse myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide was strongly immunogenic and encephalitogenic in the context of HLA-DR15 (Rich *et al.*, 2004). In another study, C57BL/6 RAG-$2^{-/-}$ mice transgenic for HLA-DR15 and an anti-MBP TCR show spontaneous paralysis, as well as epitope spread in the context of HLA-DR15 to include epitopes of MBP, MOG and αB-crystallin (Ellmerich *et al.*, 2005). Additionally, transgenic mice for HLA-DQB1*06:02, which is a part of the HLA-DR15 haplotype, show susceptibility to EAE induction by myelin-associated oligodendrocytic basic protein (MOBP) through MOBP-reactive pathogenic T cells (Kaushansky *et al.*, 2009). In a model of Goodpasture disease, animals transgenic for HLA-DR15, a risk factor of the disease, and immunized with $\alpha 3_{135-145}$ epitope show specific T cells

infiltrating the kidney, producing pro-inflammatory cytokines and resulting in Goodpasture disease (Ooi *et al.*, 2017).

While these mouse models are invaluable to gain insights into the disease, expressing a specific TCR and immunizing with a specific peptide can be limiting, especially in a complex disease such as MS. For our purpose, we wanted to generate an HLA-DR15 transgenic mouse model of the NSG background to study the effect of this molecule in human immune system-type setting and to study EBV infection in the context of this molecule.

Even though humanized mouse reconstitution leads to multilineage haematopoiesis (Traggiai *et al.*, 2004) with different levels of reconstitution, adaptive immune responses seem incomplete (Watanabe *et al.*, 2009). This is largely thought to be caused by an inefficient $CD4^+$ T cell selection on murine MHC (mMHC) class II molecules in the mouse thymus (Watanabe *et al.*, 2009). Early lymphoid progenitors which develop from hematopoietic stem cells migrate to the thymus and differentiate into $CD4^+CD8^+$ double positive cells. They then undergo positive and negative selection on self-peptides presented by the MHC molecules on thymic epithelial cells. The double positive thymocytes then differentiate into $CD4^+$ or $CD8^+$ single positive cells and repopulate peripheral lymphoid organs. This process of thymic selection is essential for a T cell repertoire that is self-tolerant, yet immunocompetent at recognising foreign antigens (Takahama *et al.*, 2010).

Interestingly, NOD $Rag1^{-/-}$ $IL2R\gamma_c^{-/-}$ animals transgenic for HLA-DR4 reconstituted with human HSC in adulthood demonstrated an increased frequency of $CD4^+$ T cell reconstitution in the blood, as well as a better human cell reconstituted thymus with human cells compared to control mice. The human T cell compartment in these mice is also functional, as observed by high levels of cytokine production after T cell stimulation. Increased B cell function was also shown, indicated by the production of all classes of immunoglobulins and the production of neutralizing anti-tetanus toxin IgG antibodies (Danner *et al.*, 2011).

In an HLA-DR1 transgenic NSG mouse model lacking expression of mMHC class II, transgene expression was observed on murine thymic epithelial cells, as well as splenic APCs. The animals reconstituted to similar levels as control NSG animals, however there was an increase in T cell reconstitution in transgenic mice, even though the T cell did not show greater TCR diversity than NSG mice. In addition, in NSG animals lacking mMHC class II expression, fewer $CD4^+$ T cells in the thymus were seen, suggesting the necessity of either murine or human MHC (hMHC) class II expression for human $CD4^+$ T cell development. There was an increase in mature naïve B cells in transgenic animals, as well as increased levels of serum immunoglobulins, indicating successful class switching probably as a result of improved interactions between HLA-DR1-restricted $CD4^+$ T cells and $HLA\text{-}DR1^+$ B cells (Goettel *et al.*,

2015). With regards to MHC class I, humanized mice transgenic for the MHC class I molecule HLA-A2 were able to elicit HLA-A2-restricted $CD8^+$ T cell responses against EBV and dengue virus, however B cell function and antibody production was not improved in these animals (Jaiswal *et al.*, 2009; Strowig *et al.*, 2009; Shultz *et al.*, 2010).

The pDOI-5 targeting vector that was used in our study to carry our transgene has been described by Kouskoff and colleagues to express foreign cDNAs in MHC class II expressing cells (Kouskoff *et al.*, 1993). In a DBAxC57BL/6 mouse model, between 10 and 30% of MHC class II-expressing cells were HLA-$DR15^+$, no aberrant expression of the transgene was seen and the number of copies of integrated transgenes ranged from 1 to 5 (Madsen *et al.*, 1999). In a study by Suzuki and colleagues, which used the pDOI-5 vector to create an HLA-DR4 transgenic NOG mouse, HLA-DR is expressed in mMHC class II positive APCs and thymic epithelial cells. Even though the percentage of positive cells from mMHC class II-expressing cells is not shown, only a fraction of these cells are HLA-DR positive (Suzuki *et al.*, 2012). With our HLA-DR15 transgenic NSG mouse, we have seen strong expression in several organs, possibly even in cells not expressing mMHC class II. As we did not perform targeted insertion, it is possible that our transgene inserted in the genome many times, as well as possibly inserting into a ubiquitously expressed promoter region, resulting in expression across many cell types. In addition, some cells seemed to be expressing HLA-DR strongly, whilst others less. Using immunohistochemistry, we were unable to dissect whether the HLA-DR signal that we see is extracellular, intracellular or secreted. We therefore aim to perform extracellular and intracellular staining of HLA-DR on mouse cells by FACS and examine protein expression by Western blot. In addition, we are trying to detect the location of our transgene in the mouse genome.

Interestingly, while some MHC class II transgenic animals still expressed mMHC class II (Danner *et al.*, 2011), others were crossed with MHC class II deficient strains (Rich *et al.*, 2004; Ellmerich *et al.*, 2005; Kaushansky *et al.*, 2009; Suzuki *et al.*, 2012; Goettel *et al.*, 2015; Ooi *et al.*, 2017). While in the former, $CD4^+$ T cells could be restricted to either murine or hMHC class II, in the latter, the T cells should be selected only in the context of the hMHC molecule. The rationale behind crossing animals to MHC class II deficient strains was not mentioned, however Suzuki and colleagues discussed that I-A sufficient animals in their study did not induce human IgG responses, as compared to I-A deficient mice. The mismatch between positive selection of human T cells by mMHC class II in the thymus and hMHC class II expressed in the periphery by B cells could be responsible for defective humoral immune responses in humanized mice. When both I-A and human transgene are present, the mMHC class II is still likely the dominant player in positive selection, as there is an abundance of

mMHC class II positive cells in the thymus. The T cell repertoire selected on the hMHC class II could hence be too small to detect (Suzuki *et al.*, 2012).

In addition, if mMHC class II is present, there could be an interspecies a/b MHC II pairing, hence enhanced positive selection (Goettel *et al.*, 2015). Because we observe strong expression across tissues, it is possible that a chain of our hMHC class II molecule paired with a chain of mMHC class II, resulting in widespread expression. While the lack of reconstitution in our transgenic mouse model is puzzling, it is possible that the transgene insertion in an important part of the genome selected a function in the remaining mouse myeloid cells that confers resistance to human hematopoietic reconstitution. Possibly the niche that is usually present in NSG mice for $CD34^+$ hematopoietic progenitor cells to occupy could be compromised, or the developing human immune cells released into the periphery could be deleted by an activated mouse myeloid system.

Nevertheless, we have started breeding our HLA-DR15 transgenic NSG mouse to an mMHC class II deficient mouse strain and will be curious to examine HLA-DR expression and reconstitution in these animals. As a back-up strain we have the BALB/c $Rag2^{-/-}$ $IL2R\gamma_c^{-/-}$ $SIRP\alpha^{NOD}$ (BRGS) mouse strain transgenic for HLA-A2 and HLA-DR15. Like NSG mice, these animals have no T or B lymphocytes, no NK cells and the 'don't eat me' signal of the NOD SIRPα allele which inhibits the phagocytic activity against human engrafted cells. These additional strains could enable us to address our remaining questions.

3.4 Methods

3.4.1 NSG-DR15 gene synthesis and cloning

The HLA-DRA*0101 α-chain was linked via a P2A linker (sequence: GGC AGC GGC GCC ACC AAC TTC AGC CTG CTG AAG CAG GCC GGC GAC GTG GAG GAG AAC CCC GGC CCC) to the HLA-DRB1*15:01 β-chain. Sequences were obtained from online sources (websites: hla.alleles.org, uniprot.org, full sequence: Appendix 2). The synthetic HLA-DR15 gene was ordered from Thermo Fisher Scientific (Invitrogen), where it was assembled from synthetic oligonucleotides and/or PCR products and inserted into the pMK-T vector backbone (Appendix 3).

To amplify the vector, it was cloned into the DH5α *E. coli* strain. Briefly, 50 µl of *E. coli* was mixed with 250 ng of vector, heat-shocked for 30 seconds at 42°C and put on ice for 5 minutes.

300 μl of LB medium was then added and the mix was incubated at 37°C for 1 hour, after which 5 ml of LB plus kanamycin (Merk Milipore) was added. After overnight incubation, the DNA was isolated with a miniprep kit (PureYield Plasmid Miniprep System, Promega Helix). Primers were designed manually (help from online tools; Appendix 4) to be: 19-22 base pairs in length, melting temperature 51.1-52.4°C, GC content 40-52.6% and ordered desalted and without further modifications (Microsynth). Full list of primers see in Appendix 5. The HLA-DR15 gene was then sequenced to verify correct sequences (Microsynth) with three primers spanning the length of the gene (Appendix 5).

EcoRI restriction enzyme (New England BioLabs) was used to digest the HLA-DR15 insert from the pMK-T vector. In a 50 μl total reaction volume, 1 μl (10 units) of EcoRI enzyme, 5 μl (10x) buffer and 1 μg of DNA was used and incubated for 1 hour at 37°C. A 1% agarose (Promega) gel was used to separate the insert from the rest of the vector. The band size 1653 bp (size of HLA-DR15 insert) was excised and DNA was cleaned from the gel as per manufacturer's instructions (Wizard SV Gel and PCR Clean-up System, Promega Helix).

The pDOI-5 vector plasmid, carrying the Eα promoter, was ordered from ATCC (ATCC 87058, (Kouskoff *et al.*, 1993), 1993, Appendix 3). To amplify the plasmid, it was cloned into the DH5α *E. coli* strain as mentioned above (ampicillin resistance instead of kanamycin) and after overnight incubation, the DNA was isolated with a miniprep kit (PureYield Plasmid Miniprep System, Promega). The Eα promoter was then sequenced to verify correct sequence (Microsynth) with three primers spanning the length of the promoter (Appendix 1 and 5). In order to insert the HLA-DR15 insert, the pDOI-5 vector was digested with EcoRI (as mentioned above, in 30 μl total volume with 700 ng DNA) and dephosphorylated with Antarctic phosphatase (New England BioLabs). After 1 hour incubation with EcoRI restriction enzyme, 1 μl (5 units) of Antarctic phosphatase enzyme with 4 μl (10x) of buffer were added to the mixture and for a total of 40 μl. After 30 minutes incubation at 37°C, the reaction was stopped by heat-inactivation at 80°C for 2 minutes and followed by a PCR clean-up as per manufacturer's instructions (Wizard SV Gel and PCR Clean-up System, Promega Helix).

To ligate the EcoRI-excised HLA-DR15 insert to the EcoRI-cut and dephosphorylated pDOI-5 vector, 50 ng of vector was added to 3-fold molar excess of insert (34.2 ng – calculated with online ligation calculator Promega or Gene link). Quick Ligation buffer (2x) and 1 μl of Quick T4 DNA ligase (New England BioLabs) was added, mixed, centrifuged briefly, incubated at room temperature (25°C) for 5 minutes and kept on ice.

Stbl3 strain of *E. coli* was used for transformation. The bacteria were mixed with 4 μl of ligation mix (rest stored at -20°C), left on ice for 30 minutes, heat-shocked for 30 seconds at 43°C and kept 5 more minutes on ice. Next, 40 μl of S.O.C. Medium (Invitrogen) was added and kept for

45 minutes at 37°C at 250 rpm and plated on LB plus ampicillin (Merck) on bacterial plates. After overnight incubation at 37°C, 10 colonies were picked and placed into 5 ml LB plus ampicillin to incubate and grow over night. 500 μl of the grown colonies was placed into 25% glycerol and stored at -80°C. Minipreps were performed on the rest of the cultures from the 10 colonies and the isolated DNA was sent for sequencing. To test whether these colonies had HLA-DR15 inserted in correct orientation into the pDOI-5 vector, three primers, used initially to sequence the Eα promoter, together with a new primer which spanned the end of Eα promoter through the β-globin gene onto the HLA-DR15 insert were used (Appendix 5). Two colonies showed correct insertion and colony 10 was used for further work.

BglI restriction enzyme (Thermo Fisher Scientific) was used to cut away bacterial components from the pDOI-5 plasmid (Kouskoff et al., 1993). In a 30 μl total reaction volume, 1 μl of BglI enzyme (10 units), 3 μl (10x) buffer and 1 μg DNA was used and incubated for 1 hour at 37°C. Dephosphorylation with Antarctic phosphatase followed (described above). The product was run on an agarose gel to separate into four bands corresponding to four BglI restriction sites on the vector. The largest band (around 5500 bp) was excised from the gel and DNA cleaned as per manufacturer's instructions (Wizard SV Gel and PCR Clean-up System, Promega Helix), with final elution in DEPC-treated H_20. The sequence was confirmed with sequencing with 6 primers spanning the Eα promoter and HLA-DR15 insert (Appendix 5).

3.4.2 HLA-DR15 mouse pronuclear injection

BglI digestion was performed until linearized DNA amounted to 10 μg, at least 80 ng/μl. DNA was given to the laboratory of Dr. Johannes vom Berg and Dr. Thorsten Buch (LTK, Zurich), who performed the pronuclear injection into the oocytes of female NSG animals.

After successful founders carrying the transgene were obtained, they were then transferred to our mouse facility in Schlieren and in Irchel and breeding colonies were initiated.

3.4.3 Genotyping PCR

For genotyping, ear biopsies were added to 100 μl of lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0, 0.2% SDS) supplemented with 2 μl (200 μg/ml) Proteinase K (Roche) and left overnight at 56°C at 550 rpm. Then, 300 μl of distilled H_20 was added and centrifuged at 12 000 rpm for 10 minutes. After centrifugation, 400 μl of the supernatant was transferred to 500 μl of isopropanol, vortexed and centrifuged at 12 000 rpm for 10 minutes. Supernatant was removed and 200 μl ice-cold EtOH added, vortexed and centrifuged at 12

000 rpm for 10 minutes. Supernatant was removed and DNA air dried for 15-30 minutes and finally resuspended in DEPC-treated H_20 (Thermo Fischer Scientific).

Genotyping PCR was done with TaKaRa LA Taq DNA polymerase (with Mg^{2+} buffer) (Clontech). In a total volume of 25 μl, 0.32 μl of each 10 μM primer (Microsynth, Appendix 5), 2.5 μl (10x) PCR mic, 4 μl dNTP mix (10 mM), 0.25 μl Takara LA Taq, approximately 15 ng of DNA and DEPC-treated H_20 (Thermo Fischer Scientific) were added. For PCR program, stage 1: 5 minutes at 95°C, stage 2: (32x) 30 seconds at 94°C, 45 seconds at 50°C and 3 minutes at 68°C. 6x DNA loading dye (Thermo Fischer Scientific) was added to the PCR product and it was loaded on a 3% agarose (Promega) gel containing 10 000x Gel Red (Biotium), in addition to 7 μl of 100 bp DNA ladder (Solis Biodyne). The gel was run at 100V for 3-4 hours.

4 Investigating the effect of MS-associated risk variants

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H.Z., B.C. and C.E. performed the experiments. B.C. prepared data for M.R.. M.R. performed logistic and beta regression analyses. C.M., B.C. and H.Z. short-listed SNPs for analysis. H.Z. optimized SNP-typing method, performed reconstitution of mice and analysis. A.Z. and R.C. determined EBV titers. C.M. and B.C. supervised and C.M., H.Z. and B.C. designed the study. C.M. acquired funding.

4.1 Introduction

MS is an immune-mediated, demyelinating disease of the CNS with a multifactorial etiology and disease mechanisms are still not completely understood (Sospedra and Martin, 2005). More than two hundred genetic risk factors, as well as several environmental risk factors, have been implicated in this disease (Olsson *et al.*, 2017; International Multiple Sclerosis Genetics, 2019). The strongest genetic risk factor is the HLA-DR15 haplotype of the MHC class II molecule (Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Patsopoulos *et al.*, 2013) and the environmental risk factor with the strongest evidence is EBV infection, which additionally synergizes with the HLA locus to further increase disease risk (Olsson *et al.*, 2017).

In the last decade, large GWAS studies replicated and identified first 110 and then 230 genetic loci implicated in the risk of MS which lie outside the MHC locus (Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Cotsapas and Genetics, 2018; International Multiple Sclerosis Genetics, 2019). The nearest gene relative to the specific SNP was identified and interestingly, these MS-associated SNPs frequently lie in regulatory regions of genes that mainly play a role in the adaptive immune response, more specifically in T cell activation and proliferation and T helper cell differentiation. The relevant genes include cytokine pathways, co-stimulatory molecules and immunologically relevant signal transduction molecules (Sawcer *et al.*, 2014). In addition, genes involved in innate immunity were also described to be involved in MS risk in the last two years (Cotsapas and Genetics, 2018; International Multiple Sclerosis Genetics, 2019). This further highlights the immune-mediated pathogenesis of MS (Sawcer *et al.*, 2014). Because the odds ratios of individual SNPs are estimated to be quite small (around 1.14-1.58), it is very unlikely that they act in isolation, as the genetic risk of MS is clearly polygenic (Bush *et al.*, 2010). For the majority of the risk variants, their causality and function remain to be investigated.

In this chapter, we aimed to develop a SNP-typing method for 16 pre-selected SNPs of interest. Then, using our humanized NSG mouse model reconstituted with SNP-typed human fetal-liver derived $CD34^+$ hematopoietic progenitor cells, we looked at the effect of the SNP rs2371108 downstream of the EOMES gene, which is a transcription factor involved in T and NK cell cytotoxicity and effector functions. In particular, we were interested to see differences in EOMES expression and EBV immune control through changes in viral loads and T cell populations isolated from PBMCs or splenocytes of infected animals.

4.2 Results

4.2.1 Pre-selection included 16 MS-associated risk variants

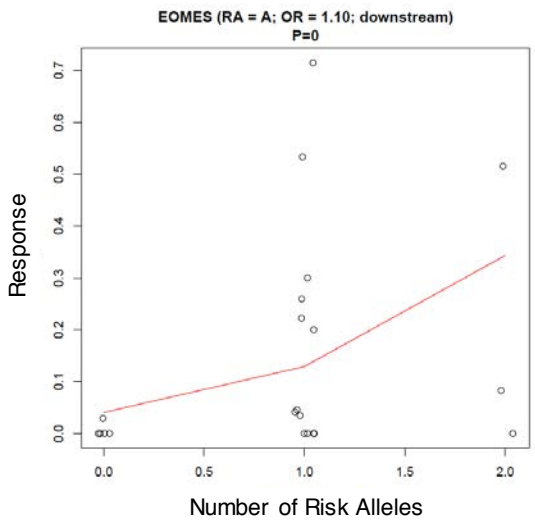
Large GWAS studies have shown that more than two hundred SNPs are risk factors for MS (International Multiple Sclerosis Genetics, 2019). Using our large dataset of past humanized mouse cohorts, we wanted to first examine whether certain SNPs correlate to the increase in activation we observe in our mice at steady state (Chapter 2, manuscript Fig. 4A). First, a selection of donors was outsourced to be SNP-typed for 110 SNPs previously identified in two GWAS studies (Sawcer *et al.*, 2011; Beecham *et al.*, 2013). Then, logistic regression and beta regression analyses were performed to examine the relationship between T cell activation and number of risk alleles present in the donor; homozygous negative for risk allele, heterozygous and homozygous positive for risk allele.

From these analyses, we have selected 16 SNPs based on the statistics obtained from the regression analyses or on general interest in the potential function of the nearby gene to have an effect during EBV infection. In the GWAS studies, the gene closest to the identified SNPs is the gene allocated to potential SNP function. We have therefore kept the nomenclature with the risk variant number (eg. rs60600003) and closest gene (eg. ELMO1) (Fig. 15A and Appendix 6). The SNP that appeared significant in both analyses and appeared most interesting was the SNP downstream of the EOMES gene (Fig. 15B and 15C). Having one risk allele was associated with elevated expression of HLA-DR in the T cell compartment in our humanized mice. The number of donors homozygous for the risk allele was too low to include that condition in the beta regression analysis (Fig. 15C).

A

Risk variant	Gene
rs3007421	PLEKHG5
rs2283792	MAPK1
rs6880778	PTGER4/DAB2
rs1021156	ZC2HC1A/PKIA
rs7120737	AGBL2
rs1886700	CDH3
rs2726518	TET2
rs1014486	IL12A
rs12927355	CLEC16A
rs60600003	ELMO1
rs2371108	EOMES
rs35929052	IRF8
rs1782645	ZMIZ1
rs1800693	TNFRSF1A
rs12296430	LTBR
rs12149527	WWOX

B Logistic regression



C Beta regression

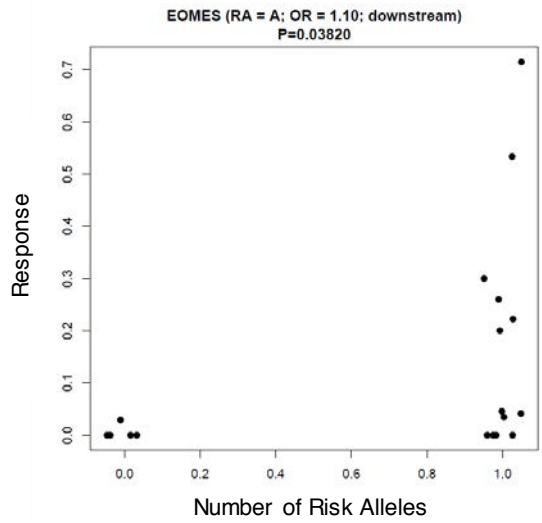


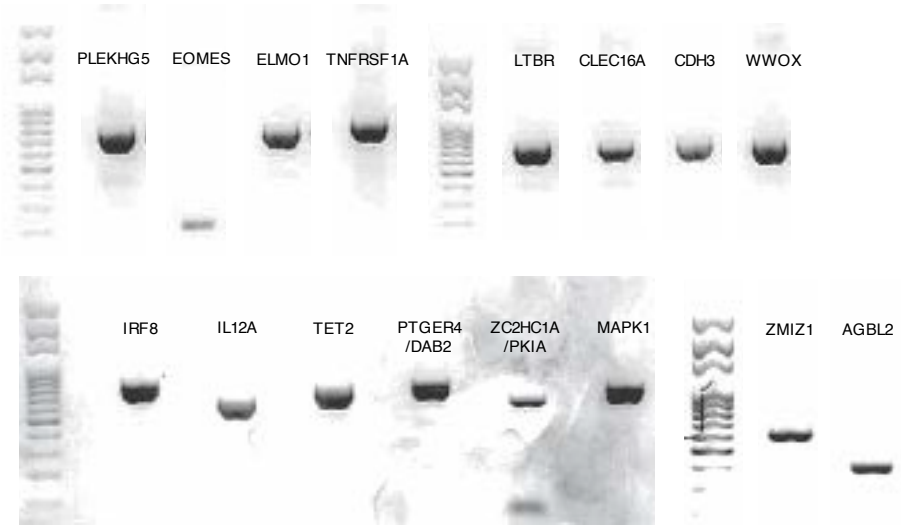
Figure 15. EOMES gene as the most interesting risk allele candidate. (**A**) List of 16 SNPs chosen based on logistic and beta regression analyses or on interest. SNPs were selected from a list of 110 SNPs published in two large GWAS studies (Sawcer *et al.*, 2011; Beecham *et al.*, 2013). 20 donors were SNP-typed and 110 SNPs correlated with increase in activation (HLA-DR$^+$) in T cell compartment in animals showing T cell activation above 20%. The SNP number and nearest gene name are shown. Logistic (**B**) and beta regression (**C**) analyses for the SNP rs2371108 downstream of the EOMES gene. Response indicates association with the increase in activation (HLA-DR$^+$) of the T cell compartment at steady state in NSG mice. Number of risk alleles indicates homozygous negative (0.0), heterozygous (1.0) or homozygous positive (2.0) for SNP. One point indicates one donor.

4.2.2 Developing a SNP-typing method

Next, we developed an in-house SNP typing method to be able to SNP-type our donors without having to outsource. We found sequences flanking the SNP of interest and designed primers around 400 base pairs on each end of the SNP (Appendix 7). We then isolated DNA from the $CD34^-$ fraction of our frozen donors, ran a PCR with the respective primers and sent the isolated DNA for sequencing (Fig. 16A). We were able to obtain clear PCR bands of each SNP area. The region around the SNP downstream of the EOMES gene is very complex and it was difficult to design primers to amplify the region efficiently. In the end, a region of around 100 base pairs amplifies well (Fig. 16B and Appendix 8). Upon matching the sequence to the original sequence obtained from online sources, we could determine if the donor was homozygous negative, heterozygous or homozygous positive for the SNP, according to the appearance of the peak at given position (Fig. 16C).

A

B



C

ELMO1 (rs60600003)	
Risk Allele	C
Immunobase Alleles	T/G
Gene	Reverse strand

Original sequence: TTTTCACTGGTGGTTTGGTA**T**GAAAAACGGCTCCCCTTGGT

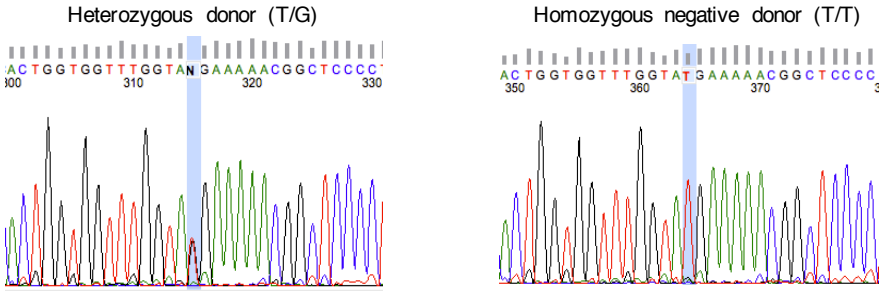


Figure 16. Optimized in-house SNP-typing protocol. (**A**) A schematic demonstrating the SNP-typing process. The sequence around each SNP was obtained from online sources and primers were designed to flank around 400 base pairs on each side of SNP. DNA was isolated from the $CD34^-$ fraction of our donors and the region around the SNP was amplified with specific primers in PCR. The correct band was then excised from the gel, DNA was isolated and sent for sequencing. (**B**) Specific bands for 16 SNPs of interest. 100bp ladders were used and the name of the closest gene to each SNP labelled above band. (**C**) Example sequencing result for SNP rs60600003. The nucleotide of risk allele was noted, as well as alleles noted in the Immunobase database and gene direction. The position of the SNP is enlarged and highlighted in a section of the original sequence. Two peaks at SNP position in a heterozygous donor and one peak in homozygous negative donor.

4.2.3 Presence of the EOMES risk variant does not lead to differences in EOMES expression in bulk T cells

Next, we wanted to investigate *in vivo* functions of these risk variants. Even though the odds ratios for individual risk variants are low (Bush *et al.*, 2010), there is evidence so far for a few SNPs regulating the proximal gene expression (Gregory *et al.*, 2012). After SNP-typing our donors, we selected which donors would be suitable for reconstitution. We performed two experiments, which included 6 different donors. The SNP-typing of these donors for our 16 SNPs of interest can be found in Appendix 9. As all the donors largely differed, we chose to first investigate one SNP of interest; the risk variant rs2371108 downstream of the EOMES gene. We reconstituted animals with donors homozygous positive (+/+), homozygous negative (-/-) or heterozygous (+/-) for this SNP and aimed to investigate EOMES expression and T cell responses.

After reconstitution, we performed EBV infection and monitored immune responses. As expected, we observed a decrease in the frequencies of total $CD4^+$ T cells (Fig. 17A) as a result of a large expansion of $CD8^+$ T cells (Fig. 17B) during the course of EBV infection, but no marked differences between the three groups of donors. Additionally, when looking at the frequencies of $EOMES^+$ $CD4^+$ (Fig. 17C) and $CD8^+$ (Fig. 17D) T cells in the blood during the infection, there appeared to be a trend in week 4 of infection with the highest frequencies of EOMES in the homozygous negative donor, however this was not anymore apparent at time of sacrifice. At around week four of infection, the frequencies of $EOMES^+$ $CD8^+$ T cells reached up to 90% and in the $CD4^+$ T cell compartment 20-30% (Fig. 17C and 17D). Higher frequencies of $EOMES^+$ $CD8^+$ T cells in the blood were seen in the homozygous positive donors at the start of experiment both in PBS and EBV-infected animals (Fig. 17D). No differences are however observed in the total numbers of both $CD4^+$ (Fig. 17E) and $CD8^+$ T cells (Fig. 17F) in the three donor groups.

In the spleen, similar patterns were observed. The frequencies of $CD4^+$ T cells expressing EOMES were lower than in $CD8^+$ T cells, where almost 100% express the transcription factor (Fig. 17G and 17H). Looking at the total numbers of these cells, no differences were observed between the donor groups for both $CD4^+$ (Fig. 17I) and $CD8^+$ T cells (Fig. 17J).

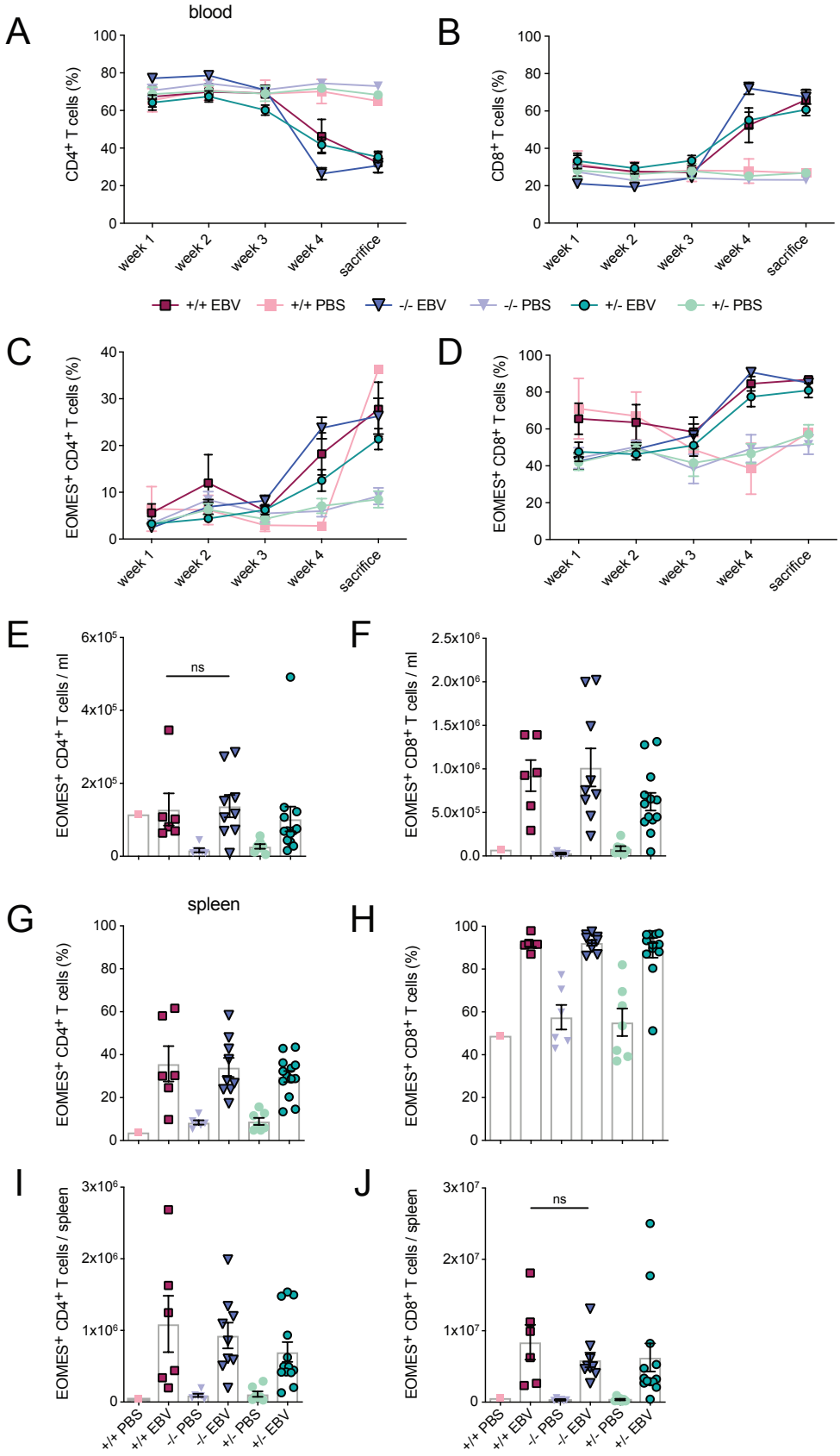


Figure 17. No difference in frequencies and total numbers in donors differing for EOMES risk variant. Animals were reconstituted with donors homozygous positive (+/+), homozygous negative (-/-) and heterozygous (+/-) for the risk variant rs2371108 downstream of the EOMES gene. Three months

after reconstitution, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4.5 weeks. The frequencies of $CD4^+$ (**A**) and $CD8^+$ T cells (**B**) and the frequencies of $EOMES^+$ $CD4^+$ (**C**) and $CD8^+$ T cells (**D**) in the length of the experiment were measured. The total numbers of $EOMES^+$ $CD4^+$ (**E**) and $CD8^+$ T cells (**F**) in the blood and frequencies of $EOMES^+$ $CD4^+$ (**G**) and $CD8^+$ T cells (**H**) in the spleen and their total numbers per spleen (**I** and **J**, respectively) were measured. Data shown are combined from two experiments and contain 1 to 13 animals per group. Non-significant comparisons are indicated and based on the Mann-Whitney U test. Each symbol indicates one animal (mean ± SEM).

As there was no difference in the frequencies or total numbers of cells expressing EOMES, we examined the protein expression of this transcription factor. For this, we analysed the median fluorescence intensity (MFI) of EOMES expressed in $CD4^+$ and $CD8^+$ T cells and then normalized it to the mean MFI value of PBS animals of the homozygous negative donor group. We have not observed differences in EOMES expression in bulk $CD4^+$ (Fig. 18A) and $CD8^+$ T cells (Fig. 18B) in blood and spleen (Fig. 18C and 18D, respectively).

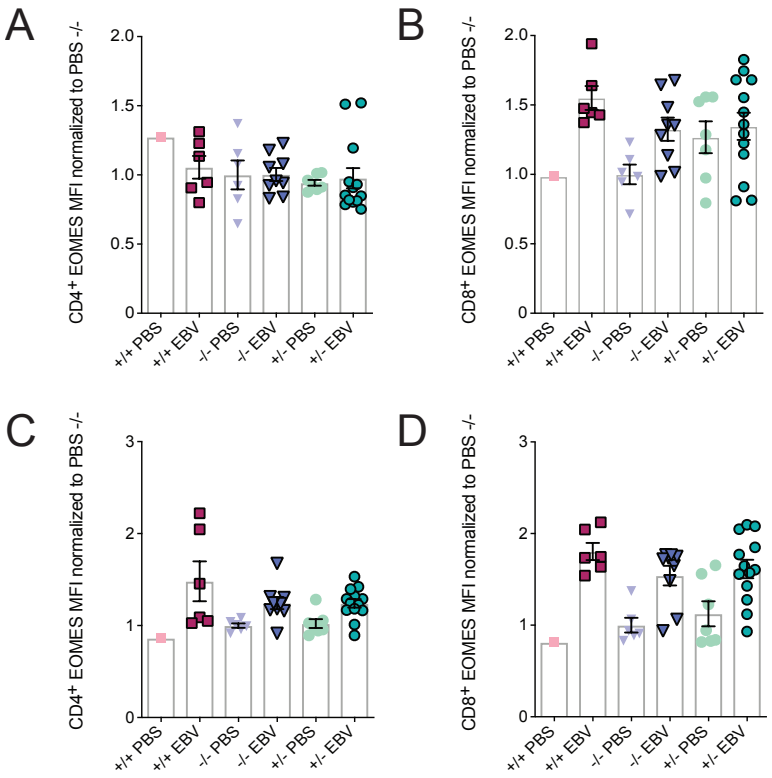


Figure 18. No difference in EOMES MFI in donors differing for EOMES risk variant. Animals were reconstituted with donors homozygous positive (+/+), homozygous negative (-/-) and heterozygous (+/-) for the risk variant rs2371108 downstream of the EOMES gene. Three months after reconstitution,

animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4.5 weeks. The median fluorescent intensity (MFI) of EOMES in $CD4^+$ (**A**) and $CD8^+$ T cells (**B**) in the blood and MFI of EOMES in $CD4^+$ (**C**) and $CD8^+$ T cells (**D**) in the spleen was measured. Each point was normalized to the average MFI of PBS animals reconstituted with the homozygous negative (-/-) donor. Data shown are combined from two experiments and contain 1 to 13 animals per group. Each symbol indicates one animal (mean ± SEM).

Finally, we examined the viral loads in the three different donor groups during EBV infection. If the risk variant was causal to the EOMES transcription factor, this could lead to a defect in T cell cytotoxicity and thus lowered EBV immune control. As expected, we observed an increase in peripheral blood viral loads from week 3 of EBV infection. Interestingly, even though at week 3, animals reconstituted with homozygous negative donors show the highest viral loads, animals reconstituted with homozygous positive donors show the highest blood viral loads at sacrifice (Fig. 19A). However, no differences are observed in the splenic viral loads between the donor groups (Fig. 19B).

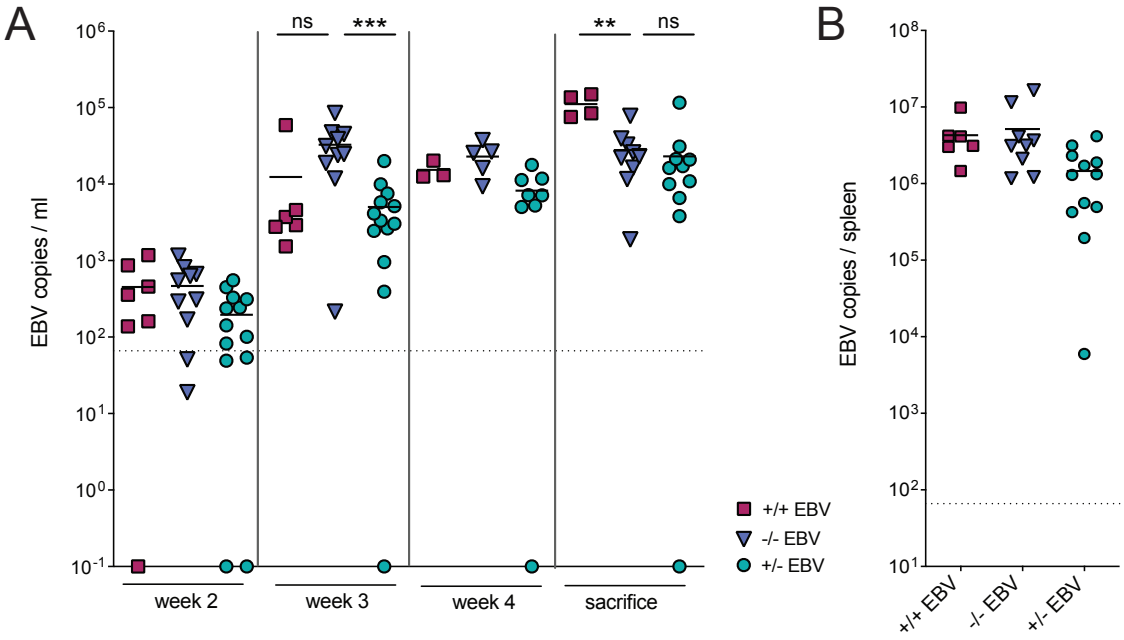


Figure 19. Higher blood viral loads at sacrifice in donors +/+ for EOMES risk variant. Three months after reconstitution, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4.5 weeks. Blood (**A**) and spleen (**B**) EBV viral loads at time of sacrifice (4.5 weeks post infection with 10^5 EBV infectious units) of animals reconstituted with donors homozygous positive (+/+), homozygous negative (-/-) and heterozygous (+/-) for the risk variant downstream of the EOMES gene.

Data shown are combined from two experiments and contain 3 to 13 animals per group. ** $p < 0.01$, *** $p < 0.001$, significant comparisons are indicated and based on the Mann-Whitney U test. Each symbol indicates one animal, full line indicates the mean and dashed line the limit of detection.

4.3 Discussion

Large GWAS have identified over two hundred risk variants outside of the MHC locus associated with MS risk (Sawcer *et al.*, 2011; Beecham *et al.*, 2013; Cotsapas and Genetics, 2018; International Multiple Sclerosis Genetics, 2019). Of these variants, while a few are predicted to be coding variants and quantitative trait loci for gene expression, all of them coincide with areas associated with regulatory function and several lie within transcription-factor binding sites within the genome, suggesting an importance of gene expression control in MS. In addition, linkage disequilibrium with other risk variants in the genome further increases chances of causal effect (Parnell *et al.*, 2014; Sawcer *et al.*, 2014). The genes closest to the SNPs interestingly play a role in the adaptive immune response, mainly in T cell activation, proliferation and T helper cell differentiation (Sawcer *et al.*, 2014), as well as innate immunity (Cotsapas and Genetics, 2018; International Multiple Sclerosis Genetics, 2019). The direct effects and roles of these risk variants are largely unknown. Understanding which risk variants are causal in general, but also causal to the nearest gene is important to better understand MS genetics, pathogenesis and gene-environment interactions, as well as to gain insights into existing therapies and develop new ones.

In this study, we developed a SNP-typing method for 16 risk variants which appeared to be associated with higher basal activation in our humanized mouse model or were of interest to us. The genes closest to these risk variants are, among others, transcription factors or their regulators, or associated with the NFκB pathway, TNF receptor family, signaling or endocytosis. As each donor has a different genetic make-up and comparing several SNPs in donors differing in many risk variants would be very challenging, we decided to focus first on one risk variant. We chose the SNP rs2371108 downstream of the EOMES gene (Beecham *et al.*, 2013) and reconstituted NSG mice with donors homozygous positive, negative or heterozygous for the EOMES risk variant.

EOMES, a T-box transcription factor similar to T-bet, is mainly expressed by activated $CD8^+$ T cells and both resting and activated NK cells (Intlekofer *et al.*, 2005) and is a key

regulatory gene involved in cell-mediated immunity (Pearce *et al.*, 2003). In $CD4^+$ T cells, EOMES is expressed by only a portion of cells at steady state, but expression increases during activation (Lupar *et al.*, 2015). Both EOMES and T-bet regulate IFNγ expression and cytotoxic molecules in $CD8^+$ T cells, such as perforin and granzyme B (Pearce *et al.*, 2003). The combined actions of these two transcription factors induce a non-redundant pathway that controls the differentiation of effector $CD8^+$ T cells. In addition, animals partially deficient for EOMES and fully deficient for TBX21, the gene encoding T-bet, showed loss of almost all IL-15-dependent cell lineages; NKT cells, NK cells and memory $CD8^+$ T cells. This was likely due changes in the ability of these cell types to respond to IL-15 (Intlekofer *et al.*, 2005). Furthermore, both T-bet and EOMES work together to sustain expression of CD122, the B-subunit of the IL-2 and IL-15 receptors, to stimulate survival and expansion of memory $CD8^+$ T cells (Intlekofer *et al.*, 2005). In chronic infections, however, EOMES expression is increased in $CD8^+$ T cells and supports their exhaustion. In addition, $EOMES^{hi}$ virus-specific $CD8^+$ T cells demonstrate a lower co-production of IFNγ and TNFα and an increase in granzyme B and cytotoxicity, even with lower levels of degranulation (Paley *et al.*, 2012).

Even though there is no published work to date investigating this specific EOMES risk variant, there has been some work done on the EOMES gene. In MS, the expression of transcription factors that control T cell and NK cell differentiation, mainly EOMES and TBX1, was found to be lower and highly dysregulated in MS patients compared to healthy controls. The expression of these two transcription factors, which share similar patterns of distribution amongst immune cells, correlated with each other, as well as with other transcription factors known to regulate T and NK cells, such as RUNX3 and TOX. Upon examining a different MS-associated SNP for EOMES, rs11129295, it was found to be associated with EOMES expression. However, the low expression of EOMES in MS is likely a combined effect of several genetic variants (Parnell *et al.*, 2014). In a later study, EOMES and TBX21 transcription factor expression correlated with HLA-DR15 genotype, but not with serum EBNA1 antibody titers when trying to predict poor EBV immune control (McKay *et al.*, 2016). Even though in one large analysis, the EOMES SNP rs11129295 was an expression quantitative trait loci (eQTL) for EOMES expression (Westra *et al.*, 2013), these results were not seen in controls or MS patients in a smaller cohort (McKay *et al.*, 2016). Thus, the effect of the SNP genotype on gene expression is not large enough to be seen in small cohorts and expression association is much better observed at the disease level rather than using SNP genotype alone (McKay *et al.*, 2016).

In a model of EAE, EOMES-deficiency yielded more $FoxP3^+$ $CD4^+$ T cells and increased protection against disease, indicating a role of the transcription factor in maintaining a balance between inflammatory and tolerance inducing responses (Lupar *et al.*, 2015).

Additionally, $CD4^+$ T cells expressing EOMES were shown to cause a late onset form of EAE and were increased in SPMS patients, compared with healthy controls or patients with RRMS. These $CD4^+$ T cells shared characteristics of cytotoxic $CD8^+$ T cells, such as granzyme B expression and degranulation measured by surface CD107a expression. Signs of CNS inflammation were reduced after siRNA blocking of granzyme B, suggesting that these $EOMES^+$ $CD4^+$ T cells could act as pathogenic lymphocytes in chronic stages of CNS inflammation (Raveney *et al.*, 2015).

Outside autoimmunity, EOMES-driven $KLRG1^+$ $CD4^+$ T cells infiltrate melanoma tumors (Curran *et al.*, 2013). In another mouse model of melanoma, double stimulation of $CD4^+$ T cells by CD134 and CD137 lead to EOMES expression which was responsible for cytotoxic function via granzyme B. The costimulatory pathway downstream of the double stimulation not only expanded Ag-specific cytotoxic $CD4^+$ T_H1 cells, but interestingly also imprinted Ag-inexperienced bystander T cells with similar functions. The double stimulation was additionally able to program $CD4^+$ T cells independently from $CD8^+$ T cells to control the murine melanoma (Qui *et al.*, 2011). Upon the recognition of self-antigen and in the absence of inflammation, $CD8^+$ T cells did not induce T-bet and EOMES and did not acquire effector functions, boosting tolerance to these antigens. However, in an inflammatory environment during LCMV infection, higher expression of EOMES, effector cytokine expression and effector differentiation was induced in autoreactive $CD8^+$ T cells (Jackson *et al.*, 2014).

Looking at bulk T cells in peripheral blood and splenocytes, we did not observe differences in frequencies or total number of $EOMES^+$ cells, nor differences in EOMES expression in animals reconstituted with the three groups of donors and infected with EBV. In fact, this SNP lies 500 bp downstream of the EOMES gene and it is possible that it is not causal to the expression of this transcription factor. This does not exclude, however, that this may affect the expression or regulation of a different gene, or of it being in linkage disequilibrium with another causal risk variant. In addition, we only studied bulk T cells and any small effect might have been masked. We will perform T cell cloning from EBV-infected animals and buffy coats and investigate EOMES expression in individual lymphoblastoid cell line (LCL)-restricted $CD4^+$ or $CD8^+$ T cell clones by FACS analysis and Western blot. Additionally, we aim to look closer at T cell subsets and differences in EOMES expression.

We have also seen differences in viral loads in our EBV-infected animals. Whether the EOMES SNP could lead to less controlled viral loads, hence possibly a stronger immune activation during acute EBV infection still needs further work. To gain an insight into this, however, we plan to perform LCL killing assays with T cell clones from homozygous positive, negative and heterozygous donors. Nevertheless, the differences in viral loads could be due

to donor variation and genetic differences which we did not compare in the study leading to differences in the immune control of EBV.

A few risk variants associated with MS risk have been studied and found to have a causal link to disease development. Homozygosity for the intronic risk allele of CLEC16A rs12927335 demonstrated higher expression of SOCS1, a negative regulator of cytokine signalling and regulation of homeostasis, and CLEC16A, a C-type lectin involved in immune regulation, in $CD4^+$ T cells, but not in $CD8^+$ T cells (Leikfoss *et al.*, 2015). The genotype for another CLEC16A risk variant rs12708716 was associated with the expression of CLEC16A transcripts in the thymus, suggesting thymus-specific regulation (Mero *et al.*, 2011). CLEC16A shows higher expression in the white matter and enhanced levels in PBMCs of MS patients than controls. Additionally, it plays a role in MHC class II expression and antigen presentation on APCs (van Luijn *et al.*, 2015). CLEC16A also has an effect on murine thymic epithelial cell autophagy, altering T cell selection (Schuster *et al.*, 2015), however, its function in T cells requires further study (Leikfoss *et al.*, 2015). Of note, this risk variant was included in the selection of risk variants in our initial analysis.

The SNP rs1800693 in TNFRSF1A does not change the extracellular expression of tumor necrosis factor receptor 1 (TNFR1), but interestingly causes the skipping of exon 6 and its absence, which leads to the receptor lacking the transmembrane and cytoplasmic domains. Therefore, the SNP leads to higher concentrations of the soluble form of TNFR1, which can block TNF and hence acts as a TNF neutralizer (Gregory *et al.*, 2012). This is an important finding, as drugs that act to block TNF have shown to lead to exacerbation of MS (van Oosten *et al.*, 1996; Arnason *et al.*, 1999). Another MS-associated causal risk variant is rs12874404 in the TNFRF13B gene, which encodes the cytokine B-cell activating factor (BAFF). It is an insertion-deletion causal variant, which yields a shorter transcript that leads to the increased production of soluble BAFF, as well as being an eQTL that increases TNFRSF13B expression. The risk variant also increased circulating B cells and levels of total IgG, IgA and IgM (Steri *et al.*, 2017).

However, identifying causal variants is challenging. The low-frequency variant rs117913124 in the CYP2R1 gene, which encodes vitamin D 25-hydroxylase, strongly associates with vitamin D levels. Heterozygous carriers for this variant have an increased risk of vitamin D insufficiency and MS. However, because of the lack of functional experiments, the exact function of this variant and causality cannot be confirmed (Manousaki *et al.*, 2017). In line with this, risk variants in the *IL7R* were confirmed to be associated with MS. The mRNA expression of both IL-7R and IL-7 in the CSF was higher for MS patients than controls, implying a pathophysiological importance of this pathway in the CSF of MS patients. However, genotype

for the risk variant rs6897932 did not correlate to IL-7R mRNA expression (Lundmark *et al.*, 2007).

With our model, we wish to replicate current findings and also investigate additional SNPs for causal effects. As only gene expression has so far been studied for the CLEC16A risk variant, we plan to examine changes in protein expression on different cell types. An additional SNP of interest to study is the intronic risk variants rs1782645 in the ZMIZ1 gene and rs2726518 in the TET2 gene. ZMIZ1 belongs to the family of protein inhibitor of activated STAT (PIAS)-like co-regulators, believed to interact with and regulate several DNA-binding transcription factors (Shuai and Liu, 2005). ZMIZ1 expression is reduced in MS, increased in response to vitamin D and weakly negatively correlated with EBNA1 antibody titers. This indicates involvement in disease susceptibility and immune dysregulation (Fewings *et al.*, 2017). TET2 proteins are involved in the epigenetic control of DNA transcription, by mediating DNA methylation. TET2 expression is down-regulated in the PBMCs of MS patients through the aberrant methylation of its promoter (Calabrese *et al.*, 2014). Interestingly, EBV infection represses TET2 mRNA and protein levels by EBV transcripts BARF0 and LMP2A. Upon the knockdown of TET2, EBV infection led to de novo methylation of several gene promoters, leading to their repression (Namba-Fukuyo *et al.*, 2016). Interestingly, TET2 mRNA and protein expression correlated with the EBV type III latency program, with the depletion of TET2 resulting in the decrease of latent gene expression and switch to the lytic gene expression program. It interacts with EBNA2 and together, they cooperate in demethylation of loci involved in EBV-driven B cell transformation (Lu *et al.*, 2017).

4.4 Materials and methods

4.4.1 Risk variant selection

HFL donors were SNP-typed for the 110 risk variants described in the GWAS studies Sawcer *et al*., 2011 and Beecham *et al*., 2013. It was next determined how many animals per cohort showed activation (staining positive for HLA-DR) above 25%, termed HLA-DR high. The analysis was done on a continuous basis using the frequencies calculated of total animals in a cohort that are HLA-DR high. To determine which risk variants correlated with increased activation, the data was analyzed by Prof. Dr. Mark Robinson. A logistic regression analysis and a following beta regression analysis were performed with cut-off $p>0.05$, displaying

increasing activation with increasing risk allele presence. Using these analyses, we selected 16 risk variants to study (Appendix 6).

4.4.2 SNP-typing

For the SNP-typing of HLA-DR15$^+$ HFL donors, the CD34$^-$ fraction was thawed and DNA isolated (DNeasy Blood & Tissue Kit, Qiagen) as per manufacturer's instructions with DNA eluted in DEPC-treated H_20 in the last step of DNA isolation. DNA yield was measured by the NanoDrop (Thermo Fisher Scientific).

The sequence 400 bp around the chosen variants (800 bp total) was found on ImmunoBase.org and verified with dbSNP (NCBI). Primers were designed manually (help from online tools; Appendix 4) to be 20-28 base pairs in length, melting temperature 58-60°C, GC content 40-60%, BLASTed (NCBI) to verify its unique position in genome and ordered desalted and without further modifications (Microsynth). Full list of primers see in Appendix 7.

In a total volume of 50 μl, 600 ng of DNA was added (800 ng for EOMES, ZMIZ1, AGBL2, ZC2HC1A), 1.25 μl of each 10 μM primer (Microsynth, Appendix 7), 0.5 μl DNA Phusion DNA polymerase (Thermo Fisher Scientific), 10 μl GC buffer from kit, 1 μl 10mM dNTPs, 3.5 μl (7%) DMSO (EOMES no DMSO) and DEPC-treated H_20. For PCR program, stage 1: 30 seconds at 98°C, stage 2: (x35) 7 seconds at 98°C, 20 seconds at 60°C (ZMIZ1, AGBL2 62°C), 25 seconds at 72°C, stage 3: 7 minutes at 72°C and hold at 10°C. 6x DNA loading dye (Thermo Fisher Scientific) was added to the PCR product and it was loaded on a 1% agarose (Promega) gel containing 10 000x Gel Red (Biotium), in addition to 7 μl of 1 kB or 100 bp DNA ladder (Solis Biodyne). The gel was run at 100V for 2 hours.

For sequencing, the band of the correct size (Appendix 8) was excised from the gel and cleaned-up to isolate DNA according to manufacturer's instructions (Wizard SV Gel and PCR Clean-up System, Promega Helix), with final elution in DEPC-treated H_20. The DNA was measured (NanoDrop) and sent for sequencing with pre-mixed 3 μl 10 μM forward primer. Sequencing .fasta files were double checked for clean peaks (FinchTV) and sequencing .ab1 files were matched to sequences 400 bp around the variant to determine the nucleotide at the SNP position. SNP-typing protocol was first verified with HFL donors that were already SNP-typed for initial analysis and risk variant selection. Upon protocol verification, further non-characterized HFLs were SNP-typed for further experiments.

4.4.3 Animal work

NOD-scid $\gamma_c^{-/-}$ (NSG) animals (Jackson Laboratory, Bar Harbor, Maine, USA) were maintained at the Institute of Experimental Immunology, University of Zürich, under specific pathogen-free conditions. Newborn pups (1-5 days old) were irradiated with 1 Gy and injected intrahepatically 5-7 hours later with 1.5-3 x 10^5 human fetal liver (Advanced Bioscience Resources)-derived and HLA-typed $CD34^+$ stem cells. Magnetic bead separation (Miltenyi Biotech) was used to separate $CD34^+$ progenitor cells, based on manufacturer's instructions (Miltenyi Biotech). Isolated cells were frozen in liquid nitrogen. After twelve weeks, tail vein bleeding was performed and peripheral blood analysed for human cells by flow cytometry. Three months old, reconstituted animals were intraperitoneally injected with 10^5 EBV Raji infectious units (RIU) or PBS and monitored for 4.5 weeks. A single donor was used to reconstitute animals in each experiment.

4.4.4 EBV

Human embryonic kidney HEK293 cells (ATCC), containing a GFP-encoding wild type EBV BACmid (p2089; kind gift from H. Delecluse) (Delecluse *et al.*, 1998), were used to produce the B95-8 EBV strain. Raji cells (ATCC) were used for virus concentrate titration and GFP-positive cells were analysed after two days by flow cytometry (FACSCanto II or LSR Fortessa, BD Biosciences) to calculate EBV RIU.

4.4.5 Flow cytometry

Collected blood was lysed using ACK lysing buffer (Gibco) and washed with PBS to obtain PBMCs. Spleens were dissociated using a 70 μM strainer and layered on Ficoll-Paque (GE Healthcare) for the separation of mononuclear cells by density gradient centrifugation. Beckman Coulter Act diff Analyzer was used to determine total numbers of leukocytes from the white blood cell count. PBMCs or splenocytes were stained with extracellular antibodies for 20 minutes at 4°C, washed and kept either in PBS or fixed in 1% paraformaldehyde (PFA). For intranuclear staining, extracellular-stained samples were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Thermo Fisher Scientific) following the manufacturer's instructions. Live/dead near IR (Invitrogen) or Zombie NIR (Biolegend) was used for live cell separation.

Antibody clones used in this study: CD4 (OKT4, RPA-T4, Biolegend), CD8 (SK1, Biolegend), EOMES (WD1928, eBioscience),

Samples were acquired on the FACSCanto II or LSR Fortessa (BD Biosciences) and analysed using the Flowjo Software (Becton, Dickinson & Company).

4.4.6 Viral load calculation

DNA from a spleen biopsy was processed using the DNeasy Blood and Tissue Kit (QIAGEN) and DNA from whole blood was extracted using the NucliSENS EasyMAG System (bioMérieux), according to the manufacturers' instructions. EBV DNA was quantified with TaqMan real-time PCR (Applied Biosystems), with modified primers for the BamH1 W fragment (5′-CTTCTCAGTCCAGCGCGTTT-3′ and 5′-CAGTGGTCCCCCTCCCTAGA-3′) and a fluorogenic probe (5′-FAM CGTAAGCCAGACAGCAGCCAATTGTCAG-TAMRA-3′). All samples were analysed in duplicates.

4.4.7 Statistical analysis

GraphPad Prism Software was used for statistical analysis and the Mann-Whitney U test was used to analyze unpaired data with a non-Gaussian distribution. A p value < 0.05 was considered statistically significant.

4.4.8 Ethics statement

The cantonal veterinary office in Zürich, Switzerland, approved all animal experimentation (protocols 148/2011, 209/2014 and 159/17). Experiments were conducted according to the Swiss Animal Welfare Act, Tierschutzgesetz (TSchG). All human samples were approved by the cantonal ethical committee of Zürich, Switzerland (protocols KEK-StV-Nr.19/08 and 2019-00837).

5 Conclusion

MS is a complex, multifactorial disease with >200 genetic risk factors implicated, as well as several environmental risk factors. The strongest genetic risk factor is the HLA-DR15 allele of the MHC class II locus and in addition, over 200 single nucleotide polymorphism risk variants contribute, albeit weakly individually, to MS risk. These risk variants are associated with genes with roles in adaptive immunity, mainly within T helper cell-related pathways. The environmental risk factor implicated in MS with strong evidence is EBV infection and interestingly, HLA-DR15 and EBV infection synergize to increase the likelihood to develop MS at least seven-fold. The mechanisms behind the interactions between the genetic risk factors and EBV infection remain unclear.

Together, our data suggest a higher basal T cell activation and impaired T cell-mediated immune control of EBV in the context of HLA-DR15, as well as an increased alloreactivity in HLA-DR15-restricted $CD4^+$ T cell clones. Upon creating an HLA-DR15 transgenic NSG mouse which would possibly have better humoral and $CD4^+$ T cell immunity, we would be able to study this further. This model would also serve as a vessel for HLA-DR15-restricted $CD4^+$ T cell clones derived from MS patients and to study their homing, reactivity and possible CNS inflammation. Finally, single risk variants confer a weak effect to MS risk, however, they likely act together in supporting pathways and also in interacting with environmental factors. When looking at the effect of the SNP downstream of the EOMES gene, we did not see an effect on protein expression at the basal level or during EBV infection in bulk T cells. We will study this further and also plan to include other SNPs in our analysis. While HLA-DR15 could be involved in immune system restriction, single risk variants could play a role in inflammation dysregulation and lowering the threshold of T cell activation. This could impact HLA-DR15-restricted effector T cell pathways and lead to defective EBV immune control, as well as heightened inflammatory settings for the selection and survival of autoreactive lymphocytes, triggering or perpetuating damage in the CNS (Fig. 20).

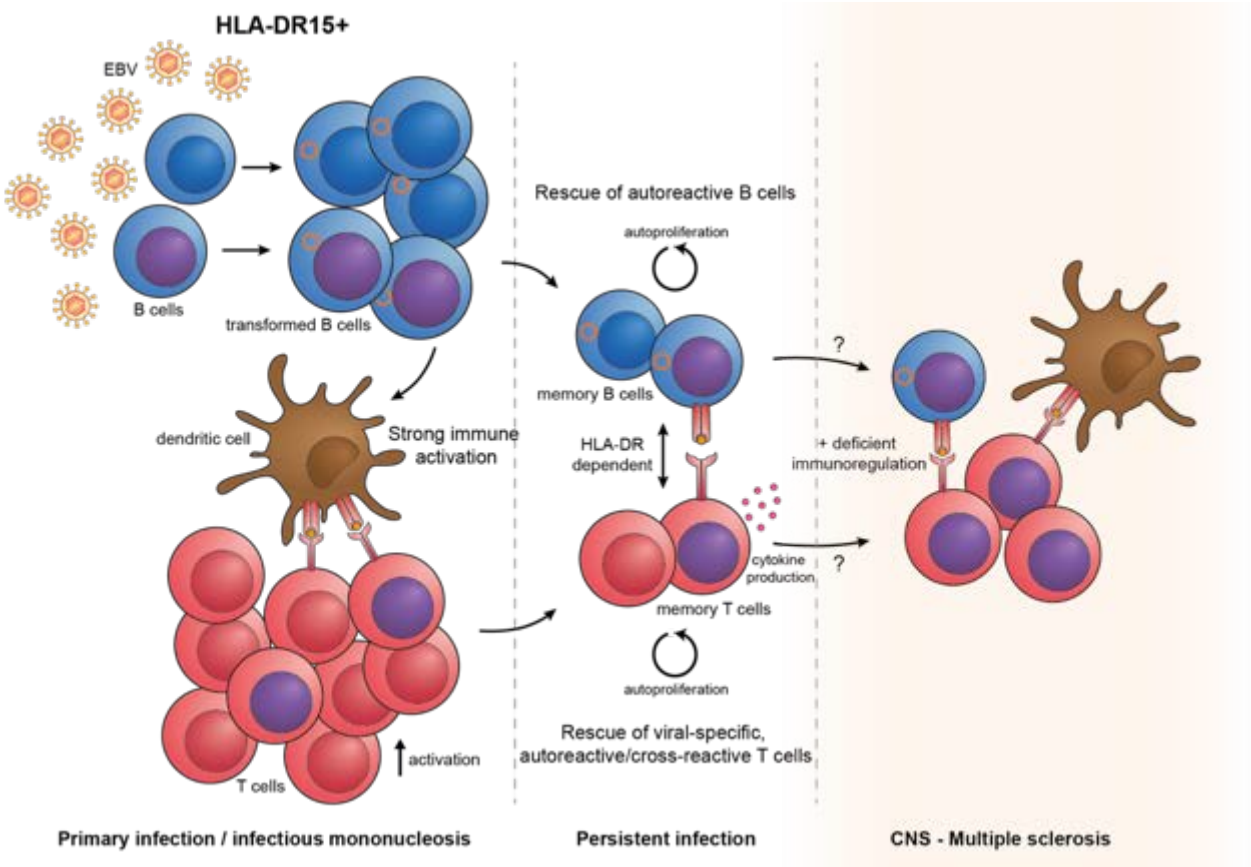


Figure 20. Suggested mechanism for the involvement of EBV infection in the development of MS. In HLA-DR15 positive donors, we see higher viral loads, as well as higher T cell activation and frequencies of $CD8^+$ T cells. EBV infection could save autoreactive (depicted as purple nuclei) B cells from apoptotic deletion and high T cell activation autoreactive or cross-reactive T cells (depicted as purple nuclei) restricted by HLA-DR15. During EBV persistence, autoproliferating EBV-infected autoreactive B cells could stimulate cross-reactive T cells in an HLA-DR dependent manner and maintain their survival. Activated, autoreactive lymphocytes could home to the CNS, where B cells might act as APCs to cross-reactive T cells and trigger CNS inflammation.

(Adapted from (Ascherio *et al.*, 2012).

6 Appendix

6.1 Full sequence of Eα promoter

TAGAGCAAAAGAACAGATGGGGATATGATACTGAGCAGCTCAGCATTCGGGGGAGAGGGAGAAGG
GAAGCTAAAATGGAAGAACTTGGATCAAACAAACGTAAAATGCACTGAAGCCCCAGAACTGATCTG
GGTCCAGTGTGGATGCACTCATGGTCACGGAGCTGTGGGAGAATGAAGTCAGATGACCGGTAAAG
AATATGGATATAGATGATAGTCATGTTCGTATAATTCAGTAATCTTTAGAATACTCCCATTT**GCCTCT**
GAGGCTCAGTTCTCCCACCCTGAACACACAGTTTTGAGTGTGGATACAAGCTGTGTGGTTTGGGTG
GCAGAGAGAAAATGGACCTGTGTCATGTGGTGAGCAAGCTGTCACAGGCTTGAACATTGGCAAGCA
GGCATTGGTGACGTCCTCAGCAGATGATGTTCCCTTGTGTCCTGTGGGCTTTGATGAAACTATGAC
ATCAGCGGGACTTCCCAGAGGCCACTGACCACTTTAGATTAAAAGTCGACCCTGACCAGTGCTCTG
CCAGCCTTAAGCTATACTTCTTGTAACATATTCAATTCTGTTTTCTGGAGGACCCATGACAGTAGCTC
ACCACGGTTTAATGTCACCAAGGATACATTATTCAGGTGGCTTCAGAAATCCCCTCAGACACACAGG
AAGCTATACTTTGGGAGAACAGGAAACCACGAACAGTGTGCCAGAGACTTCTCCCCATCACAGAGA
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GAAAACAATATTTTCCAGTGGCCCTGGAACCTTGGGCTGAACTGACCATGTTGGTGTCTAGCCCAC
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GCCTTCACACTCAGAGGTACAAATCCCCATTTTTCATATTAGCGATTTTTAATTTATTCTAGCCTCAC
TGATGTGTTTCAGATAGAACTTAGATTGGGACAGAAGATTGTGTATTTTACAACCAACATTCCCAATC
TCTTGAAATTTTTGTCCTGTTTGTCTACAGCCTTTATTATTTTTTTGTTAATAAGTGGAAAAATTTCTTC
TTGAGGAAAATTATTTCTTGAAATGTTAAGTGGAAACTCGGATACTAAATAGGACCTGGTTGCAAGG
AACCCTTTCCTAGCAACAGATGTGTCAGTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCTTT
GGATTTTAATCCCTTTTAGTTCTTGTTAATTCTGCCTCAGTCTGC

BglI site

6.2 Full sequence of HLA-DR15

ATATAT**GAATTC**ATGGCCATAAGTGGAGTCCCTGTGCTAGGATTTTTCATCATAGCTGTGCTGATGAGCG
CTCAGGAATCATGGGCTATCAAAGAAGAACATGTGATCATCCAGGCCGAGTTCTATCTGAATCCTGACCA
ATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGATTTTCCATGTGGATATGGCAAAGAAGGAGACG
GTCTGGCGGCTTGAAGAATTTGGACGATTTGCCAGCTTTGAGGCTCAAGGTGCATTGGCCAACATAGCT
GTGGACAAAGCCAACCTGGAAATCATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCA
GAGGTAACTGTGCTCACGAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGAC
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CACCTGCCAAGTGGAGCACCCAAGCGTGACAAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAAT
CTGCACAGAGCAAGATGCTGAGTGGAGTCGGGGGCTTTGTGCTGGGCCTGCTCTTCCTTGGGGCCGG
GCTGTTCATCTACTTCAGGAATCAGAAAGGACACTCTGGACTTCAGCCAACAGGATTCCTGAGCTGA**GA**
ATTCTATATA

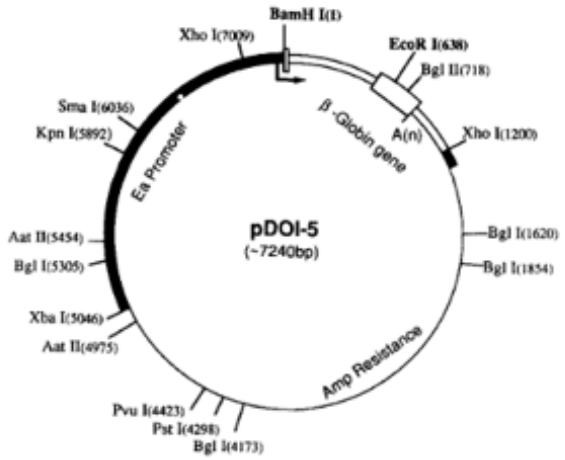
DRA

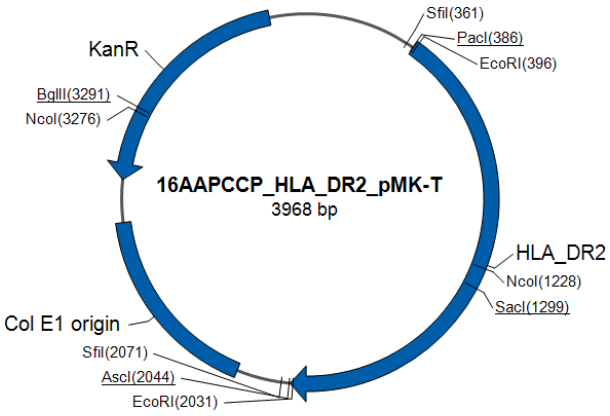
P2linker

DRB

EcoRI site

6.3 Vectors (pMK-T and pDOI-5)





6.4 Online tools SNP primer design

Melting Temperature (Tm) Calculation - www.biophp.org/minitools/melting_temperature/demo.php

Reverse complement – www.bioinformatics.org/sms/rev_comp.html

DNA/RNA GC Content Calculator – www.endmemo.come/bio/gc.php

6.5 List of primers DR2 mouse

Purpose	10 μM forward primer	10 μM reverse primer
Sequence Eα promoter and beginning of rabbit β-globin gene on pDOI-5 plasmid	EαP1_F GAACAGATGGGGATATGATACT	-
	EαP2_F ATACTTTGGGAGAACAGGAAAC	
	EαP3_F TGTGCCCAAGCTATTCTTTAGT	

	EαP4_F GAGTGCTTTGGATTTTAATCCC	
Sequence HLA-DR15 gene in pMK-T vector and after insertion into pDOI-5 plasmid	DR2 1_F GAATTGAAGGAAGGCCGTC	
	DR2 2_F CTACAGAGAACGTGGTGTG	
	DR2 3_F CACAGGCCTGATCCAGAAT	
	DR2 1_F in β-globin gene * TCTCTTTCCTACAGCTCCTG	
HLA-DR15 mouse genotyping	hDRA1U F CTGGCGGCTTGAAGAATTTGG	hDRA1U R CATGATTTCCAGGTTGGCTTTGTC

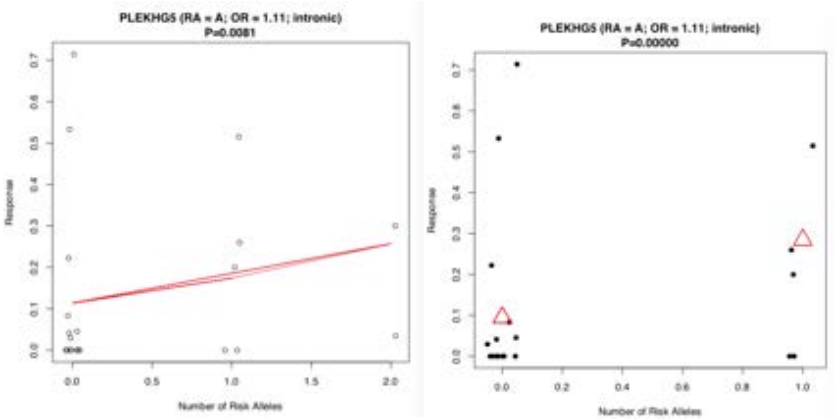
* once the gene is in the pDOI5 plasmid, the DR2 #1_F won't bind anymore as it is before the EcoRI site on pMK-T plasmid

6.6 Logistic and beta regression

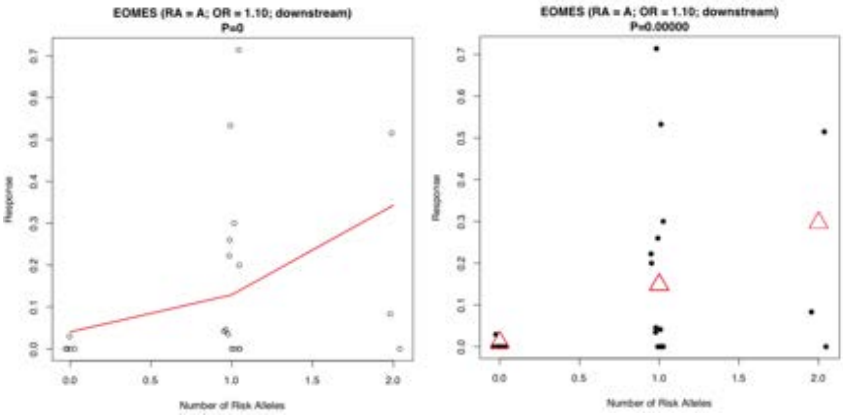
Risk variant	Gene	p logistic regression	p Beta regression
rs3007421	PLEKHG5	0.00000	0.34231
rs2283792	MAPK1	0.00016	0.90093
rs6880778	PTGER4/DAB2	0.00000	0.03625
rs1021156	ZC2HC1A/PKIA	0.00000	0.06543
rs7120737	AGBL2	0.00476	0.4162
rs1886700	CDH3	0.00291	0.19918
rs2726518	TET2	0.61089	0.09516
rs1014486	IL12A	0.00003	0.08676
rs12927355	CLEC16A	0.00352	0.47443

rs60600003	ELMO1	0.00002	0.17386
rs2371108	EOMES	0.00000	0.0382
rs35929052	IRF8	0.00000	0.11503
rs1782645	ZMIZ1	0.00001	0.6269
rs1800693	TNFRSF1A	0.00000	0.86966
rs12296430	LTBR	0.01126	n/a
rs12149527	WWOX	0.00000	0.15527

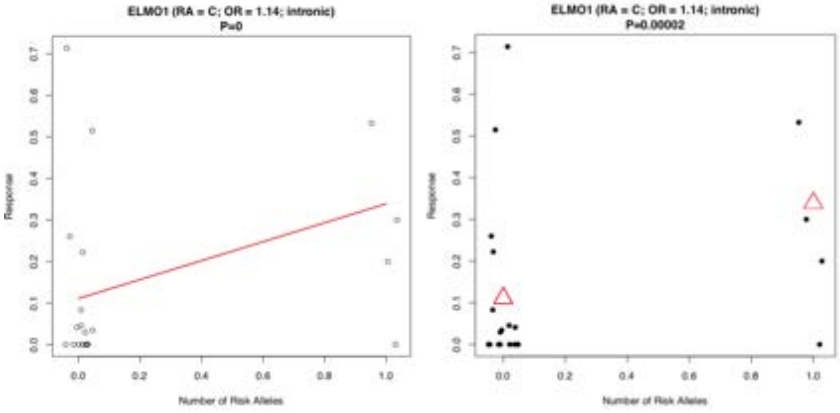
PLEKHG5



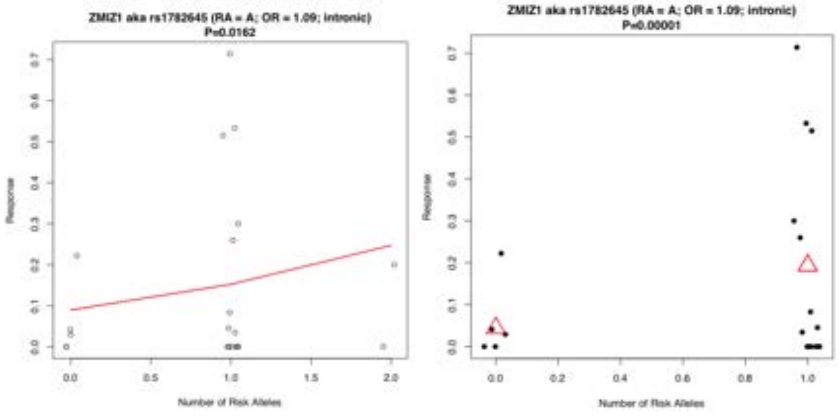
EOMES



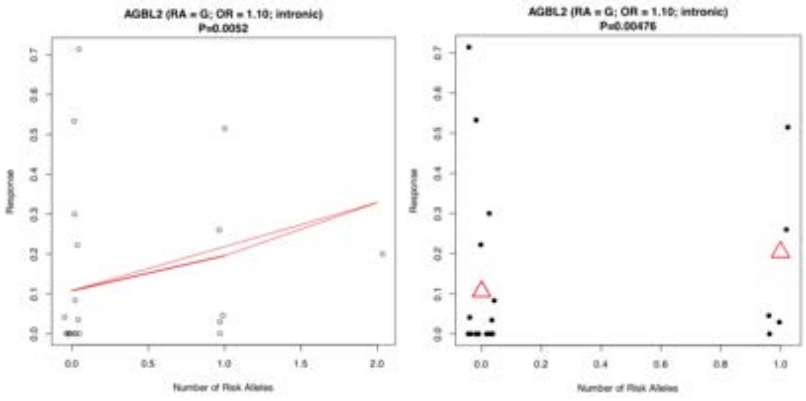
ELMO1



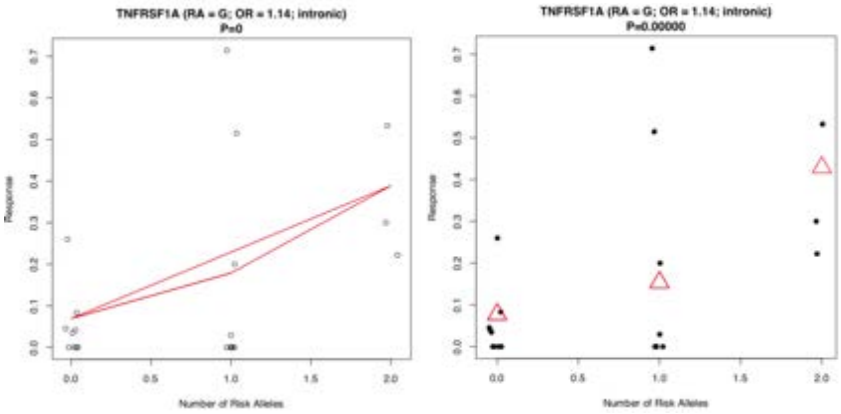
ZMIZ1



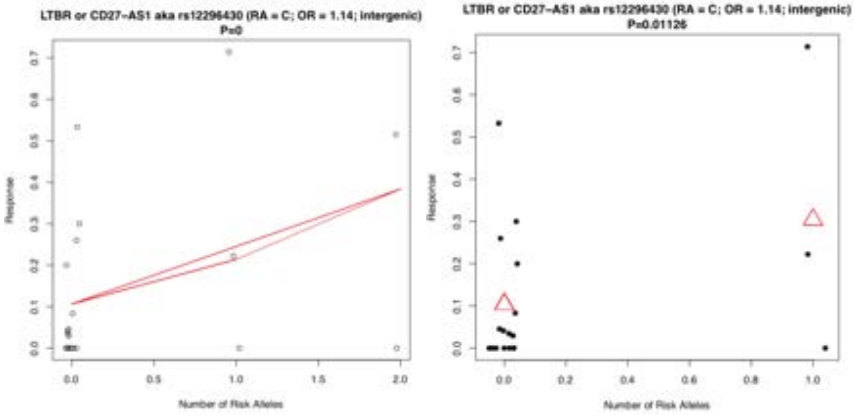
AGBL2



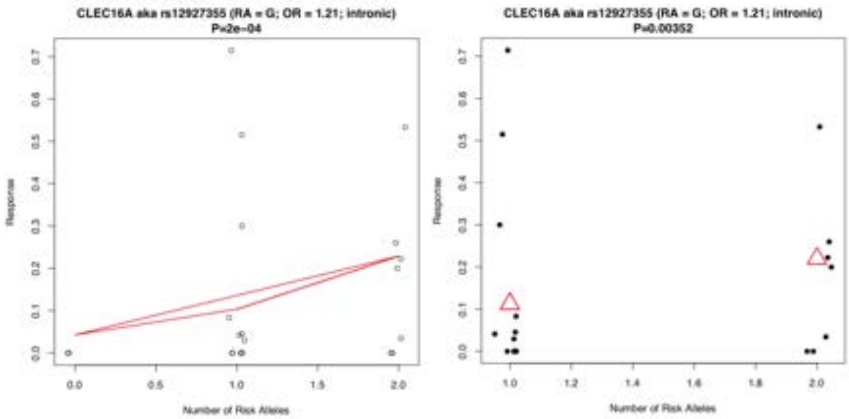
TNFRSF1A



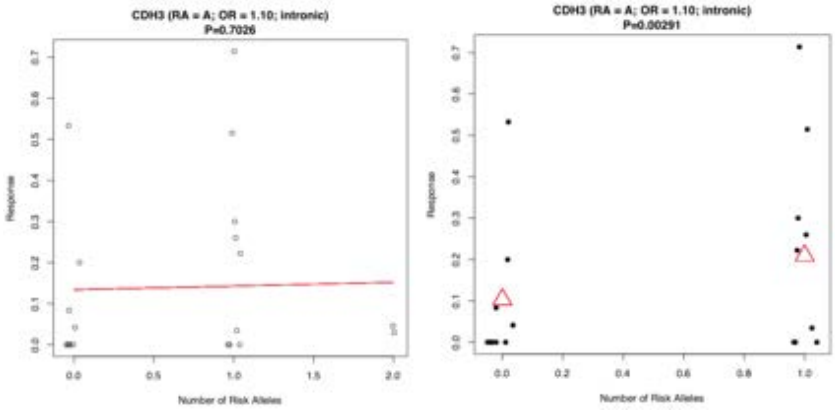
LTBR



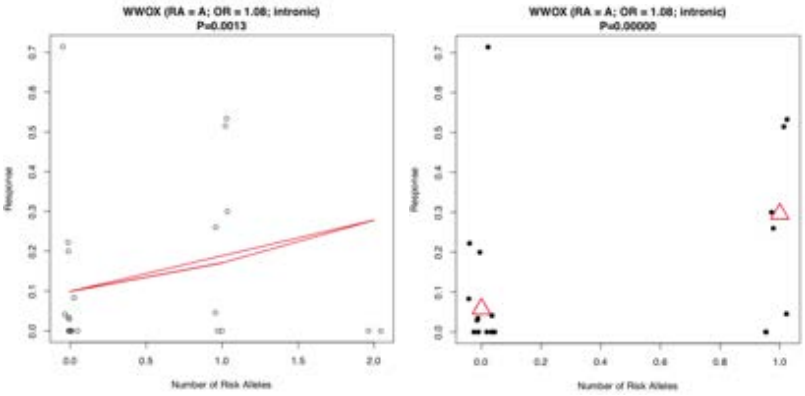
CLEC16A



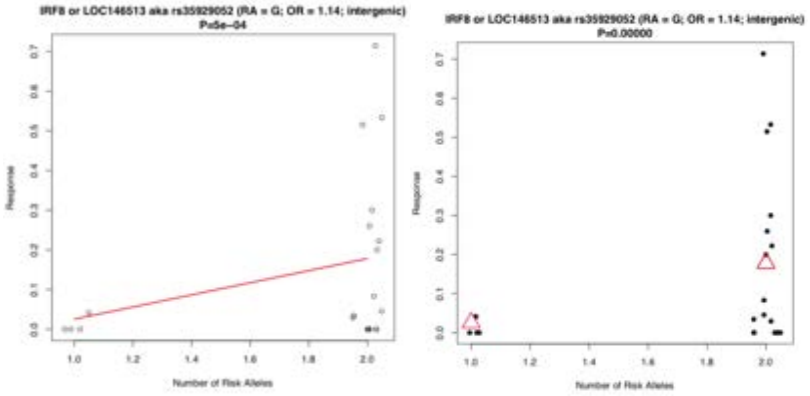
CDH3



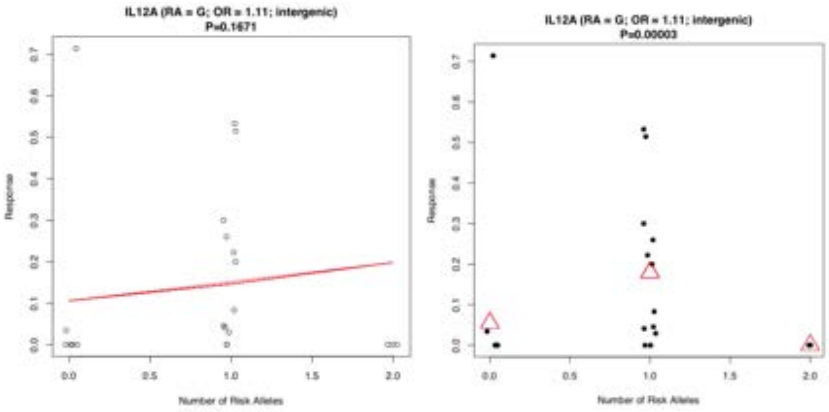
WWOX



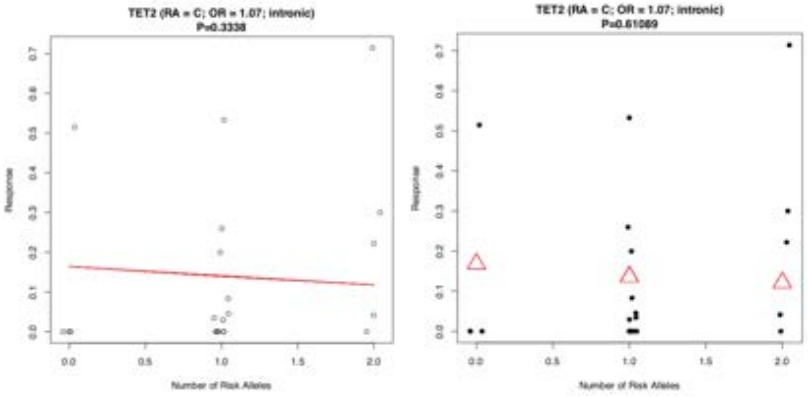
IRF8



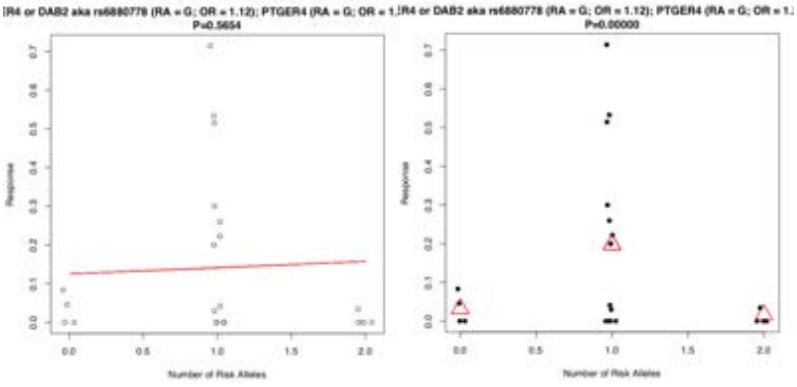
IL12A



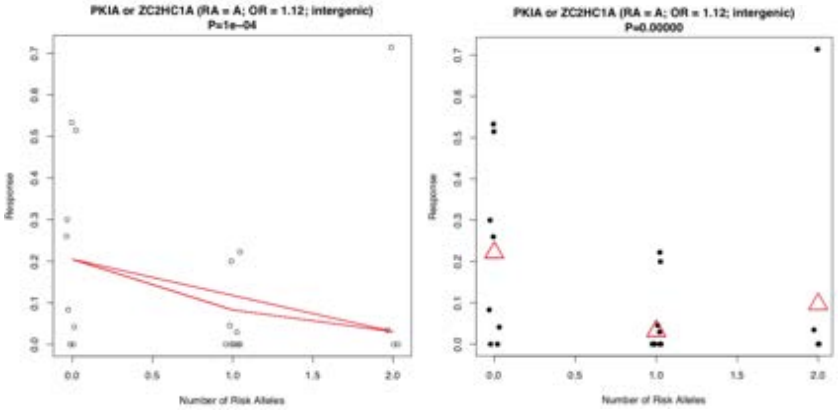
TET2



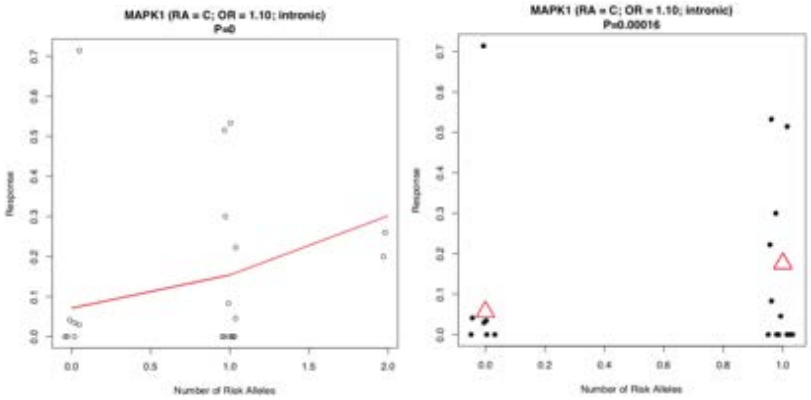
PTGER4/DAB2



ZC2HC1A/PKIA



MAPK1



6.7 List of SNP primers

Risk variant gene	10 μM forward primer	10 μM reverse primer
PLEKHG5	5’ ACAGCAGATCCGTGAAGAGGAAGC 3’	5’ TGTGCTGGGCATCTCACTGAGGAT 3’
EOMES	Primer 5F 5’ AGAAGCTGAGCTGTGTGCAG 3’	Primer 9R 5’ CCGATTGCCCACATCAGACTTAACCAA 3’
ELMO1	5’ CCCATCCATCACTGTATGTGCTACAG 3’	5’ CACTGCTTCCTGACCATGACTTCCT 3’
ZMIZ1	Primer 3F 5’ CTAGAATTTTGTGACTTGGTGAGGGGG 3’	Primer 3R 5’ CCCACCCCGATCTCTCATGTACAT 3’
AGBL2	Primer 3F	Primer 3R

	5’ TAGCCTGACGTTGTGGCATGTGC 3’	5’ TGAGATGGTGCATGGAGTGCACTTC 3’
TNFRSF1A	5’ AGCTCGACATCTCCCCAGCCAT 3’	5’ GGTTAATGGCTTGCCTAGATTCACACT 3’
LTBR	5’ GCAATGGCAGTTGACAATGGGGTAAC 3’	5’ AGTTTATTGGCTCCTGGGATTGGGG 3’
CLEC16A	5’ ATGGAGGGACTTGGACCTGACTC 3’	5’ GGACCCATCAACAAGGGTGGCTTA 3’
CDH3	5’ CCCACTCTCAAACAGGTTACTGGC 3’	5’ TGAGAGCACCCTGCACTGGGTAA 3’
WWOW	5’ GATTTCTTACTATCTTGGGCCTTCTTGG 3’	5’ CTTAGGGAATTGATGATCGGCTACTGT 3’
IRF8	5’ GTGGATGACCGTCCTTTTCAAGGAG 3’	5’ AATTCAAGAGCCAAGAGAGTATTTTGCCC 3’
IL12A	5’ CAATTCAAGATAGTCTGACCATGCTACC 3’	5’ AAAGGACTTGGCAGACGTGGGAAG 3’
TET2	5’ GAGGACTTTCGCATCTCTGTTGATAAG 3’	5’ CCAGTCTGAAAAGGCTCCATATACTGT 3’
PTGER4/DAB2	5’ GGTGTCAACAGCCATTGGATATCTACC 3’	5’ GAAGATGTCCTCTGTGGATTGTCACAC 3’
ZC2HC1A/PKIA	Primer 2F 5’ GTAGGTTATTCCTCTTCTGGTCATGGAA 3’	Primer 2R 5’ GTTGAGTACCTTCTATGCTTCAAGTCCA 3’
MAPK1	5’ TGCTATACGTGTTTGTCACACAGACATC 3’	5’ TGAAATGGAAAGTGGAGACAGAAACGAC T 3’

6.8 SNP band sizes

Risk variant gene	Band size (bp)
PLEKHG5	800
EOMES	240
ELMO1	752
ZMIZ1	563

AGBL2	363
TNFRSF1A	791
LTBR	799
CLEC16A	769
CDH3	782
WWOW	742
IRF8	731
IL12A	575
TET2	655
PTGER4/DAB2	715
ZC2HC1A/PKIA	595
MAPK1	694

6.9 SNP-typing of 6 donors SNP-EBV1 and 2

Risk variant gene	Mar-31-2016 HZ R1	Mar-08-2016 HZ R2,4,8	Mar-01-2016 HZ R3	Sept-01-2015 HZ R5	Sept-15-2015 HZ R6	Oct-07-2015 HZ R7
PLEKHG5	+/-	-/-	+/-	+/-	-/-	-/-
EOMES	+/+	-/-	+/-	+/-	+/+	+/-
ELMO1	-/-	-/-	-/-	-/-	-/-	-/-
ZMIZ1	+/-	+/-	-/-	+/-	-/-	+/-
AGBL2	-/-	-/-	na	na	na	na
TNFRSF1A	-/-	-/-	+/-	+/+	-/-	+/-
LTBR	+/+	+/+	+/+	+/+	+/+	+/+
CLEC16A	+/+	+/+	+/+	na	+/+	na
CDH3	-/-	-/-	-/-	-/-	+/+	+/-
WWOW	+/-	+/-	+/-	-/-	-/-	-/-

IRF8	+/+	+/+	+/-	+/+	+/+	+/+
IL12A	na	+/-	+/-	-/-	+/-	-/-
TET2	+/+	+/-	+/+	-/-	+/+	+/-
PTGER4/DAB2	+/-	+/-	-/-	-/-	+/+	-/-
ZC2HC1A/PKIA	na	-/-	-/-	-/-	+/-	+/-
MAPK1	+/-	+/+	+/+	+/-	-/-	-/-

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8 Curriculum Vitae

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PUBLICATIONS

Zdimerova H, Murer A, Engelmann C, Raykova A, Deng Y, Gujer C, Rühl J, McHugh D, Caduff N, Pezzino G, Capaul R, Zbinden A, Ferlazzo G, Lünemann JD, Martin R, Chatterjee B*, Münz C*. Attenuated immune control of the Epstein Barr virus in the context of the main genetic risk factor for multiple sclerosis. *Submitted manuscript*.

Chatterjee B, Deng Y, Holler A, Nunez N, Azzi T, Vanoaica LD, Müller A, **Zdimerova H**, Antsiferova O, Zbinden A, Capaul R, Dreyer JH, Nadal D, Becher B, Robinson MD, Stauss H, Münz C. CD8+ T cells retain protective functions despite sustained inhibitory receptor expression during Epstein-Barr virus infection in vivo. *PLoS Pathog*. 2019 May 30;15(5):e1007748.

McHugh D, Caduff N, Murer A, Engelmann C, Deng Y, **Zdimerova H**, Zens K, Chijioke O, Münz C. Infection and immune control of human oncogenic γ-herpesviruses in humanized mice. *Philos Trans R Soc Lond B Biol Sci*. 2019 May 27;374(1773):20180296.

Zdimerova H, Albert ML, Ingersoll MA. Harnessing the host immune response to infection – BCG immunotherapy for bladder cancer. Review Book Chapter. Infection and Cancer: Bi-Directional Interactions, M.R. Shurin, Y. Thanavala, N. Ismail. Springer Publ., NY, 2015.

CONFERENCES ATTENDED

ACTRIMS Forum 2019 – February 28-March 2 2019, Dallas, Texas USA
Poster Presentation

MSParis2017 – 7th Joint ECTRIMS-ACTRIMS Meeting – October 25-28 2017, Paris, France
Poster Presentation

Clinical Research Priority Programs, Multiple Sclerosis (CRPP^{MS}) International Symposium "Heterogeneity of Autoimmune Diseases" – June 12-13 2017, Zürich, Switzerland
Poster Presentation

Clinical Research Priority Programs, Multiple Sclerosis (CRPP^{MS}) retreat – November 29 2016, Zürich, Switzerland
Oral Presentation

17th International Symposium on EBV and associated diseases – August 8-12 2016, Zürich, Switzerland
Poster Presentation

International Workshop on Humanized Mice – January 28-30 2016, Zürich, Switzerland
Poster Presentation

CRPP^{MS} retreat – October 19 2016, Zürich, Switzerland

Miltenyi Biotec, EATI and SITC "Natural and therapy-induced anticancer immunosurveillance: the Immunoscore" – July 3-4 2014, Paris, France

Institut Pasteur Immunology Department Retreat – June 2-4 2014, Cluny, France
Poster presentation

REFERENCES

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